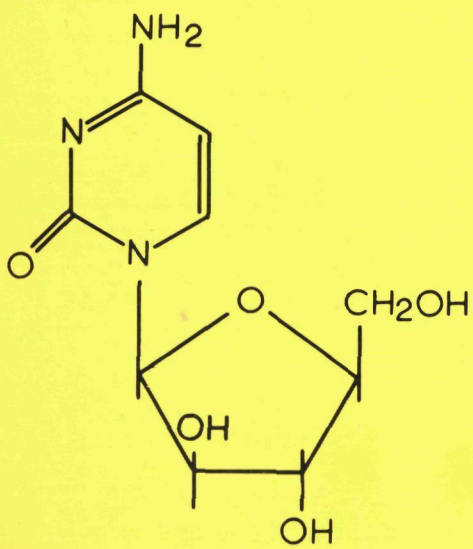
PHARMACOKINETICS OF CYTOSINE ARABINOSIDE IN ACUTE MYELOID LEUKAEMIA



H. C. VAN PROOIJEN

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF.DR.A.J.H.VENDRIK VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP 14 JANUARI 1977 DES NAMIDDAGS TE 4 UUR.

DOOR

HENDERIKUS CORNELIS VAN PROOIJEN
GEBOREN TE SASSENHEIM

KRIPS REPRO-MEPPEL

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Aan Ans en mijn ouders

Parts of this thesis are reported in the following papers:

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II Prooijen, H.C. van, Kleyn, E. van der, Haanen, C. (1976). Pharmacokinetics of cytosine arabinoside in acute myeloid leukaemia. Clin. Pharmacol. Ther. In the press.

III Prooijen, H.C. van, Vierwinden, G., Wessels, J., Haanen, C. (1977). Cytosine arabinoside binding to human plasma proteins. Arch. int. Pharmacodyn. In the press.

IV Prooijen, H.C. van, Kleyn, E. van der, Haanen, C. (1977). Pharmacodynamics of cytosine arabinoside in patients with acute myeloid leukaemia and the results of therapy. Abstract. Brit. J. Haemat. In the press. Presented at the combined meeting of the Nederlandse Vereniging voor Hematologie and the British Society for Haematology, 15th - 16th Oct. 1976.

The papers I, II, III are presented in chapter 3, 4, 5 respectively. Paper IV is presented in the chapters 6 and 7.

CONTENTS

CHAPTER I

OUTLINE AND OBJECTIVES OF THIS STUDY

Acute myeloid leukaemia (AML) is characterized by an accumulation of immature myeloid cells in the bone marrow and by a decreased production of mature granulocytes. The aetiology of the arrest in maturation of the myeloid cells is unknown. Treatment of AML is aimed at an eradication of the immature myeloid cells by cytostatic drugs. The choice and dosage schemes of the various chemotherapeutic agents have, until now, been empirical. Research into drug metabolism and the kinetics of leukaemic cells in relation to pharmacodynamics is necessary to improve the results of cytostatic treatment on a more rational basis.

Cytosine arabinoside (Ara-C) is the drug of choice in the treatment of AML. In combination with other cytostatic drugs the incidence of complete remission is at most 50 - 60 % (table I). To explain the high percentage of failures, the fate of Ara-C in individual patients has been investigated.

Ara-C is either phosphorylated intracellularly to its triphosphate (Ara-CTP) or deaminated to uracil arabinoside, a non-cytotoxic metabolite. Only in the form of Ara-CTP is the drug active as an inhibitor of cell growth and DNA synthesis. Large variations in phosphorylating and deaminating enzymatic activities have been found in the leukaemic cells of AML patients. Clinical studies even suggest that the pretreatment level of deaminating activity in the leukaemic cells determines the response of the patients to Ara-C. The differences in the intracellular Ara-CTP content not only depend on the deaminating and phosphorylating *enzymatic*

Regime	% complete remission	Reference
Ara-C + thioguanine	65 %	Clarkson (1972)
Ara-C + daunorubicine	50 %	Crowther et al.(1973)
Ara-C + daunorubicine + vincristine	55 %	Rosenthal and Maloney (1972)
Ara-C + cyclophosphamide + vincristine + prednisone	55 %	Whitecar (1972)
Ara-C + vincristine + adriamycin	75 %	Mathé et al.(1976)

Table I.

Incidence of complete remission obtained in acute myeloid leukaemia using Ara-C in combination with other cytostatic drugs.

activities, but also on the time period during which a *minimal effective plasma drug concentration* is maintained. In most treatment schemes Ara-C is administered by intravenous bolus injections. The effective plasma concentration is the resultant of the dosage and the elimination rate of the drug. The plasma elimination rate of Ara-C has been determined in several studies in which half-life values ranging from 3 to 30 minutes were found. The most important studies about Ara-C are reviewed in Chapter 2. The aim of our study was to investigate the pharmacokinetics of Ara-C in relation to the results of remission inducing therapy in AML patients. For this study a sensitive and reliable assay had to be developed since in the therapeutic range very low

plasma drug concentrations were expected, especially since the half-life of Ara-C is rather short. This method, a bio-assay, is described in Chapter 3. With this assay the half-life of Ara-C was determined and related to the results of remission inducing therapy in fourteen patients (Chapter 4). Since protein binding might affect the distribution of Ara-C, the amount of drug bound to plasma proteins was also studied (Chapter 5). The finding that the half-life of Ara-C was related significantly to the result of remission inducing therapy, led to the development of a predictive test based on the inhibition of tritiated thymidine incorporation into leukaemic bone marrow cells after injection of Ara-C (Chapter 6). The degree of inhibition reflects the amount of Ara-CTP present in the cell. The relation between the inhibition of tritiated thymidine incorporation, the half-life and the intracellular enzymatic activities is discussed (Chapter 7).

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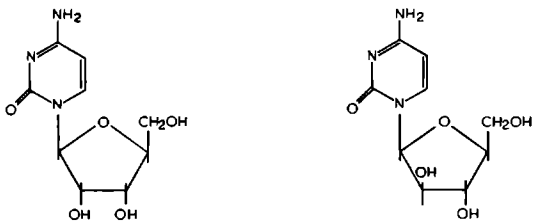
CHAPTER 2

BIOCHEMISTRY AND PHARMACOLOGY OF CYTOSINE ARABINOSIDE

2.1 MODE OF ACTION OF CYTOSINE ARABINOSIDE

2.1.1. Introduction

The compound cytosine arabinoside (Ara-C, 1-β-D-Arabinofuranosyl-cytosine) is an analogue of the naturally occurring nucleoside cytidine (fig 2.1). The drug is synthesized according to principles outlined by Cohen (1966). It is a fairly stable constituent that retains 90 % of its potency after storage at neutral pH at room temperature for 6 months (Notari et al., 1971). Unlike related derivates such as thymine arabinoside and uracil arabinoside, which have been isolated from a Caribbean sponge (Bergman et al., 1951, 1955), the drug is not known to occur naturally.



Cytidine Cytosine Arabinoside

Fig 2.1 *Structure of Cytidine and Cytosine Arabinoside*

The biological activity of Ara-C has been studied in bacteria by Slechta et al. (1961). Relatively high Ara-C concentrations (> 2 mM) were necessary to inhibit the growth and respiration of E.coli. The cytostatic properties of Ara-C were demonstrated by its growth inhibiting effect in a variety of transplantable mouse tumors including Sarcoma 180, Ehrlich ascites carcinoma, Leukaemia L-1210 and Lymphoma L5178y (Evans et al., 1961, 1964), giving a high incidence of "cures" (Wodinsky et al., 1975). In more detailed studies with bacteria and cell cultures it appeared that the inhibition of growth was related to the inhibition of DNA synthesis (Lark et al., 1964; Silagi, 1965). Treatment of pregnant rats with Ara-C, followed by administration of labeled thymidine, showed a profound inhibition of DNA synthesis in the embryos (Ritter et al., 1971).
Many studies have been undertaken to elicit the mode of action by which the drug inhibits DNA synthesis. These studies are briefly discussed.

2.1.2. Some relevant data about structure and synthesis of DNA.

In order to put the inhibitory action of Ara-C on DNA synthesis in perspective, a short review of DNA synthesis is given.

Structure of DNA

The DNA molecule consists of complementary strands of polynucleotides. Each nucleotide is composed of a pyrimidine or purine base, a sugar molecule and phosphoric acid. The pyrimidine bases are thymine (T) and cytosine (C) and the purine bases are adenine (A) and guanine (G). The sugar molecule is deoxyribose.
The backbone of each strand consists of the sugar molecules linked together through phosphate diester bonds. The bases are covalently linked to the sugars. Between the bases of the two strands hydrogen bonds can be formed i.e. adenine with thymine and guanine with cytosine. The DNA strands are complementary to each other due to this base pairing (fig 2.2).

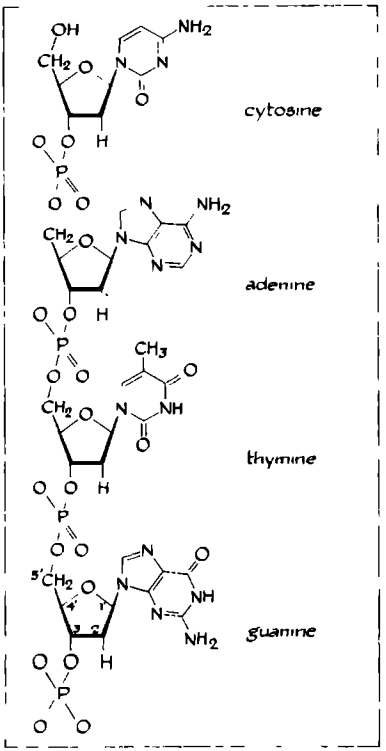


Figure 2.2
Polynucleotide strand of the DNA molecule.

Synthesis of DNA

During DNA synthesis the nucleotides are formed in the cell. Initially ribose is phosphorylated at carbon atom 5', then the bases are attached to the sugar to form the ribonucleoside 5'-monophosphates. After further phosphorylation to ribonucleoside 5'-diphosphates the ribose molecule is reduced by a ribonucleoside diphosphate reductase to deoxyribose. These reduced diphosphates are again phosphorylated to deoxyribonucleoside 5'-triphosphates (5'-triphosphates).

In early studies it has been found that *Ara-C is an inhibitor of ribonucleoside diphospate reductase* (Chu et al., 1962, 1965). DNA synthesis starts with the unwinding of the two strands of the DNA molecule. Each strand serves as a template for a newly formed polynucleotide strand. The 5'-triphosphates approach their complementary mates on the single parent strands, are held in place loosely by base pairing and are then covalently linked to the free 3'-hydroxyl group of the sugar in the growing daughter chain to form the backbone of the DNA molecule. This condensation of 5'-triphosphate is catalyzed by DNA polymerase. This enzyme holds the nucleotides, uses the strands of unwound parent DNA as templates and guarantees the specificity of the insertion of the new nucleotides. Thus at the end of DNA synthesis pairs of two identical DNA molecules exist in the cell, each consisting of one parent and one daughter strand.
Since Ara-C is also phosphorylated to its triphosphate, it has been suggested that inhibition of DNA synthesis might occur after *incorporation of the drug into DNA, resulting in cessation of chain growth* (Graham et al., 1970a; Momparler, 1972). In later reports it has been suggested that the drug inhibits DNA synthesis by *inhibiting the enzymatic activity of DNA polymerase* (Kimball et al., 1968; Momparler, 1972).

2.1.3. Inhibition of the ribonucleoside diphosphate reductase by Ara-C.

An essential step in the biosynthesis of deoxycytidine triphospate (dCTP), a precursor molecule in the process of DNA synthesis, is the reduction of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP).
Chu and Fischer (1962) incubated murine L-5178y lymphoblasts with labeled uridine. They found that in the presence of Ara-C the intracellular level of dCDP was significantly reduced, while the level of CDP was not affected. From these data it was assumed that the mode of action of Ara-C on DNA synthesis could be due to an inhibition of CDP reduction to dCDP by phosphorylated derivates of Ara-C.

Moore and Cohen (1965) investigated the effect of phosphorylated Ara-C on CDP reduction in crude enzyme extracts from Novikoff- and Ehrlich ascites tumor cells. They did not find any significant inhibition in the reduction of CDP to dCDP, either by diphosphates or by triphosphates of Ara-C.
Skoog and Nordenskjold (1971) measured the intracellular pools of deoxyribonucleotides in mouse embryo cells in the presence of Ara-C They found a transient decrease in the dCTP pool and increased levels of deoxyribosyladenine triphosphate, deoxyribosylguanine triphosphate and deoxyribosylthymine triphosphate.
From these studies it is obvious that the inhibition of CDP-reductase is not a major step in the inhibition of DNA synthesis by Ara-C.

2.1.4. Inhibition of DNA synthesis by cytosine arabinoside incorporation into DNA.

Ara-C is rapidly phosphorylated to its triphosphate and incorporation of the agent into DNA might be expected. Indeed, small amounts of radioactivity in the acid insoluble fraction (presumably DNA) have been found when various cell lines were incubated with tritiated Ara-C (Chu et al., 1965; Silagi, 1965). In some of these studies it was demonstrated by enzymatic degradation of the nucleic acid that the incorporated radioactivity really originated from Ara-C (Chu et al., 1968; Momparler, 1972). Despite the evidence that Ara-C is incorporated into DNA, the problem remains as to how this incorporation relates to inhibition of DNA synthesis.
Graham and Whitmore (1970a) incubated mouse L-cells with labeled Ara-C. After specific enzymatic digestion of DNA they found that more than 70 % of the radioactivity was released as a 3'-Ara-CMP. These data indicate that Ara-C is incorporated into the DNA chain between other nucleotides. The incorporated Ara-C may cause alterations in the DNA structure leading to a decreased activity of DNA polymerase
Momparler (1972) studied the incorporation of ^{32}p Ara-CMP in a cell free system using denaturated DNA. His data. obtained after

specific enzymatic digestion, suggest that Ara-CMP is incorporated at the end of a growing DNA chain, causing termination of further chain growth.

The relation in mouse L-cells between inhibition of DNA synthesis and loss of cell viability in the presence of different Ara-C concentrations was studied by Graham et al. (1970b). They found that 3.6 x 10^{-7}M Ara-C could inhibit DNA synthesis by more than 97 % for nearly 14 hours without significant effect on cell viability. Exposure of cells to concentrations of 7.2 x 10^{-6} M caused, in addition to inhibition of DNA synthesis, an irreversible loss of viability.
From these results it can be concluded that Ara-C has a cytostatic effect at very low concentrations. At these concentrations DNA synthesis is inhibited initially but is restored after some hours and the cells multiply again in culture. At higher concentrations the drug is cytocidal.

2.1.5 Inhibition of DNA polymerase by Ara-C.

During DNA synthesis the 5'-triphosphates are incorporated into the newly formed polynucleotide strand. The incorporation is enzymatically controlled by DNA polymerase as described in paragraph 2.1.2. In crude preparations of DNA polymerase, extracted from Ehrlich ascites cells, it was found that the enzyme was inhibited by Ara-CTP (Kimball et al., 1968). Similar results were found with DNA polymerase extracted from calf thymus (Furth et al., 1968) and human leukaemic cells (Inagaki et al., 1969). Graham and Whitmore (1970) reported that in DNA polymerase extracts from mouse L-cells, Ara-CTP was a competitive inhibitor of the enzyme. The affinity of this enzyme for dCTP and Ara-CTP is about equal; in all these studies the inhibition of DNA polymerase by Ara-CTP could be reversed by dCTP.
These data indicate that Ara-CTP competes with dCTP for the active sites on DNA polymerase. This was confirmed in a cell-free system using a synthetic polynucleotide chain consisting of alternating

adenine and thymine nucleotides as template. Ara-CTP did not produce any detectable inhibition of the incorporation of labeled thymidine triphosphate in the polynucleotide strand. (Momparler, 1972).

Conclusion:

Intracellularly, Ara-C is phosphorylated to Ara-CTP. In this form the drug is active as an inhibitor of DNA synthesis. Three possible mechanisms of action of the drug have been proposed: the inhibition of DNA polymerase, the incorporation of Ara-C into DNA and the inhibition of ribonucleoside diphosphate reductase. The inhibition of DNA polymerase by Ara-CTP is undoubtedly the major site of action of the drug. The amount of Ara-C incorporated into DNA is very low but is a consistent finding and may be related to irreversible damage of the cell. It is generally accepted that the inhibition of ribonucleoside diphosphate reductase is not a significant factor in the mode of action of Ara-C.

2.2 PHARMACOLOGY OF CYTOSINE ARABINOSIDE

2.2.1. Introduction

The concentration of a drug in the direct environment of the receptors and the affinity for these receptors determine the effect produced by the drug. The drug concentration near the receptors is governed by the drug concentration in the tissue and the plasma. After oral or intravenous administration the drug is distributed from the plasma over the entire body. The rate of exchange of drug between plasma and tissues depends on the tissue perfusion, the tissue volume and the partition of the drug between plasma and tissues. On the basis of similarities in blood flow the various tissues may be grouped together and considered as compartments. All compartments are directly or indirectly in contact with the blood. The plasma and the highly vascularized tissues which rapidly exchange the drug, may be considered as one,

so called *central compartment*. The other tissues may be considered as one or two *peripheral compartments*. The drug concentration in the tissues does not correlate completely with the plasma concentration due to differences in partition and/or binding of drug to the various tissue components.
Shortly after intravenous injection the drug is distributed over the plasma and the tissues of the central compartment.The virtual volume of distribution of this compartment may be different for different drugs and depends on the amount of tissue to which the drug has access. The course of the plasma disappearance curve after an intravenous bolus injection represents the processes of distribution and elimination of the drug. Since the body can be considered as a central compartment with one or two peripheral compartments, the semilogarithmic plasma concentration curve is not a straight line but is bi- or triphasic.
In acute leukaemia the primary site of disease is the bone marrow, which is a highly vascularized tissue and therefore belongs to the central compartment. Thus differences in elimination of Ara-C in patients may correlate with clinical response.

2.2.2. Pharmacokinetics of cytosine arabinoside.

The half-life of cytosine arabinoside

Many pharmacological studies on distribution and metabolism of Ara-C have been performed with the tritium labeled drug. In all these studies the levels of radioactivity in body fluids were determined followed by chromatographic separation of Ara-C from its metabolite Ara-U. The plasma disappearance curve appeared to be biphasic with half life values for the second phase varying from 5 to 30 minutes (Creasey et al., 1966; Finkelstein et al., 1970). For pharmacokinetic studies in a large number of patients, bioassays have been developed for the determination of Ara-C in plasma using sensitive cell lines or micro-organisms. Pitillo and Hunt (1967) described a method using an actinobolin resistant strain of Streptococcus faecalis. Hanka et al. (1970) modified

this assay system and could then measure Ara-C levels as low as 0.1 mg/l.
These assays, however, were not sensitive enough for pharmacokinetic studies in man. Baguley and Falkenhaug (1971) reported a bioassay using mammalian cell lines. The test was based on the inhibition of DNA synthesis in suspensions of L-cells or mouse spleen cells. Ara-C plasma concentrations as low as 0.04 mg/l could be detected. In a later report the sensitivity of the test was increased using murine tumor cells (L1210). With this test plasma half-life values ranging from 3 to 15 minutes were measured. Although the experimental conditions were not stated, the authors reported that a poor bone marrow response was found in patients with short half-life values (Baguley et al., 1975).

Elimination of cytosine arabinoside

Pharmacological studies of Ara-C have shown that the drug rapidly disappears from the plasma after intravenous administration (Creasey et al., 1966; Momparler et al., 1972). In the liver the drug is transformed to uracil arabinoside (Ara-U) by the enzyme cytidine deaminase (Camiener, 1967). It could be demonstrated, using labeled Ara-C, that 86 % - 96 % of the radioactivity recovered in the urine was present in the form of Ara-U (Papac et al., 1965; Creasey et al., 1966).
Ho et al.(1971) recovered 80 % of the radioactivity in the urine within 24 hours of administration of labeled Ara-C; 8 % was present as Ara-C, 72 % as Ara-U. These studies indicate that Ara-C is eliminated by deamination and glomerular filtration.

2.2.3. Intracellular metabolism of cytosine arabinoside

The plasma disappearance curve of Ara-C after an intravenous bolus injection appears to be biphasic which is typical for the distribution of a drug in a two compartment open model with elimination only from the central compartment (Wagner, 1975). The first, rather rapid phase mainly represents the distribution of

drug over the two compartments while the second phase represents the elimination of the drug. The half-life values of the second phase range from 5 to 30 minutes, representing a short exposure of the cells to the drug (Creasey et al., 1966; Finkelstein et al., 1970; Baguley et al., 1971, 1975). In various cell lines it has been shown that the drug is transported from the extracellular fluid into the cell by a carrier mediated diffusion process which increases the rate of uptake (Kessel et al., 1968; Mulder et al., 1975). The enzymatic processes in the cell responsible for the activation and inactivation of the drug and their relevance for the result of treatment are discussed below.

Phosphorylation of cytosine arabinoside

Phosphorylation of Ara-C was found necessary for the inhibition of DNA synthesis and cell growth (Chu et al., 1962). In drug resistant cell lines of L1210 and 5178y it was found that phosphorylation of both deoxycytidine and Ara-C was decreased by over 90 % compared with the same drug-sensitive cell lines (Chu et al., 1965; Schrecker et al., 1968). It was concluded that phosphorylation of Ara-C was mediated by deoxycytidine kinase and that the decrease in the activity of this enzyme was responsible for resistance to the drug.

Deoxycytidine phosphokinase is a naturally occurring enzyme which catalyzes the first phosphorylation step to deoxycytidine 5'-monophosphate and cytosine arabinoside 5'-monophosphate respectively. The biochemical properties of this enzyme were first studied in crude extracts of calf thymus (Durham et al., 1968; Momparler et al., 1968). It appeared that this enzyme is rate determining in the conversion of Ara-C to Ara-CTP.

The second phosphorylation step is catalyzed by cytosine monophosphokinase (Sugino et al., 1966; Schrecker, 1970) and the third step by non-specific nucleoside diphosphokinase (Nakamura et al., 1966). In man deoxycytidine kinase has been found in all dividing cells with the highest activity in the spleen (Ho et al., 1973).

In crude extracts of AML bone marrow blast cells (Tattersall et al., 1974) and intact AML peripheral and bone marrow blast cells (Ho et al., 1973) a wide range of phosphorylating activity has been found. In a recently published study the biochemical properties of partially purified deoxycytidine phosphokinase from leukaemic blast cells were determined (Coleman et al., 1975). The enzyme demonstrates a greater affinity for deoxycytidine than for Ara-C. Of particular interest was the relatively weak inhibition by Ara-CTP and the strong inhibition by deoxycytidine and deoxycytidine triphosphate with regard to the phosphorylation of Ara-C. From these data it can be concluded that high intracellular levels of cytosine arabinoside triphosphate have no effect on further drug phosphorylation.

The uptake and in-vivo phosphorylation of Ara-C in host tissue and leukaemic cells in murine leukaemia (L1210) has been studied by Chou et al., 1975). One hour after intraperitoneal injection of labeled Ara-C the amount of free Ara-C in blood, small intestine and leukaemic cells was equal. However, the Ara-CTP of the leukaemic cells versus blood was more than 700 fold, while the Ara-CTP ratio of the small intestine versus blood was 24 fold. The inhibition of DNA synthesis was higher and more pronounced when higher levels of Ara-CTP were found in the cell. This study indicates that the differences in phosphorylation capacities between various cell types determine the chemotherapeutic activity of the drug.

Dephosphorylation of cytosine arabinoside

The rate of cytosine arabinoside triphosphate dephosphorylation was not well studied. It is assumed that the nucleoside triphosphatases and diphosphatases are high in activity compared with the monophosphatases. Thus, the rate determining dephosphorylation step is the Ara-CMP to Ara-C conversion (Schrecker, 1970). The rate of dephosphorylation of Ara-C nucleotides could be a factor in the response to Ara-C. However, in drug sensitive and

drug insensitive tumors no difference in the monophosphatase activity was found (Drahovsky et al., 1970; Schrecker, 1970).

Deamination of cytosine arabinoside

The inactivation of Ara-C by an enzyme catalyzed deamination was first reported to occur in Escherichia Coli (Pizer et al., 1960). The enzyme, cytidine deaminase, is naturally occurring and deaminates cytidine and Ara-C to uridine and uracil-arabinoside (Ara-U) respectively. In man a wide variation exists between the enzyme activities in different tissues. Highest activity has been found in the liver (Camiener, 1967; Ho, 1971).
The biochemical properties of purified preparations of cytidine deaminase obtained from normal peripheral granulocytes and peripheral leukaemic blast cells have been studied by Chabner et al. (1974). They found that the affinity of the enzyme for its physiologic substrate cytidine was higher than for Ara-C. They also found that the concentration of enzyme per mg protein was significantly higher in the normal peripheral granulocytes (3.52 $\pm$ 1.86 x 10^3 U/mg protein) than in the leukaemic blast cells from AML patients (0.19 $\pm$ 1.17 x 10^3 U/mg protein). After separation of normal bone marrow cells into mature and immature fractions by Ficoll density centrifugation the enzymatic activities were determined in each fraction. It appeared that the cytidine deaminase activity in lysates of the mature fraction was 3.55 - 14.2 times greater than the activity found in lysates of the immature fractions. The authors stated that the amount of cytidine deaminase activity is related to the process of granulocyte maturation. A potent inhibitor of cytidine deaminase is tetrahydro-uridine (THU) (Camiener et al., 1968; Mulligan et al., 1968; Ho et al., 1971; Chabner et al., 1974).

2.2.4. The relation between intracellular enzymatic activities and resistance to cytosine arabinoside.

The phosphorylation of Ara-C to its triphosphate is an essential

precondition to its function as inhibitor of DNA synthesis. In both human and mouse leukaemic cells the response to Ara-C has been correlated with the ability to accumulate cytosine arabinoside triphosphate (Kessel et al., 1969; Chou et al., 1973). Resistance to this agent may be the result of a decreased phosphorylating activity (Chu et al., 1965; Creasey et al., 1968) and/or an increased deaminating activity (Steuart et al., 1971; Chabner et al., 1974). Thus the therapeutic efficacy of Ara-C under pharmacological conditions will be influenced by a) the total amount of drug transported into the target cells and b) the relative proportions of the drug activated by phosphokinases (K) and inactivated by deaminases (D) expressed as a K/D ratio.

A wide range in the ability of intact peripheral and bone marrow leukaemic blast cells to phosphorylate or deaminate the drug has been found (Ho et al., 1973). These findings were confirmed in partially purified enzyme extracts of peripheral blast cells from AML-patients (Chabner et al., 1974; Coleman et al., 1975).

The broad range of the deaminating and phosphorylating abilities of AML cells is reflected in the variability of the calculated K/D ratio.

It appeared that the K/D ratio in peripheral blast cells was of little value in predicting the response of AML patients to Ara-C (Coleman et al., 1975; Smyth et al., 1976).

The large variation in the phosphorylating ability of the blast cells may be due to the large individual differences in the percentage of proliferating cells in the bone marrow as well as in the peripheral blood (Hillen et al., 1975).

The deaminating activity of the blast cells in the bone marrow seems to be important. In clinical studies Steuart and Burke (1971) suggested that the pretreatment level of cytidine deaminase in the blast cells of AML patients determines the response to Ara-C.

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CHAPTER 3

A SENSITIVE BIO-ASSAY FOR PHARMACOKINETIC STUDIES OF CYTOSINE ARABINOSIDE IN MAN

ABSTRACT

A sensitive bio-assay is presented for the determination of plasma levels of cytosine arabinoside (Ara-C). The test is based on the inhibition of the incorporation of tritiated thymidine into DNA of rat bone marrow cells in the presence of Ara-C. The accuracy and the sensitivity of the test is demonstrated. Unknown plasma concentrations of Ara-C as low as 0.003 mg/l can be measured.
The plasma disappearance curve of Ara-C after an intravenous bolus injection in a dose of 100 mg/m^2, is biphasic.
The half-life values of these two phases vary for individuals which is illustrated in two patients with acute leukaemia in which the plasma half-life of Ara-C in the first phase was 1.8 and 1.4 minutes, and in the second phase 16.0 and 9.2 minutes respectively.

3.1 INTRODUCTION

The pyrimidine analog cytosine arabinoside (Ara-C) has proved to be effective in the treatment of acute myeloblastic leukaemia (AML) in man. The therapeutic effect of Ara-C strongly depends on the dosage and the way of administration. Complete remission is obtained in more than half of the patients treated with Ara-C in combination with other drugs like thioguanine (Clarkson, 1972) or daunorubicin (Crowther et al., 1973).

To achieve optimal treatment schedules, knowledge of pharmacological parameters, such as plasma half-life and effective drug-concentration in relevant tissues, is required. Baguley et al. (1971) report a significant correlation between a short half-life of Ara-C in plasma and a poor response.

The drug is eliminated by glomerular filtration and by enzymatic deamination to uracil-arabinoside (Ara-U). Between 87% and 91 % of the radioactivity recovered in the urine after intravenous administration of tritiated cytosine-arabinoside (^{3}HAra-C) appeared to be Ara-U (Creasey et al., 1966). The enzyme responsible for deamination of Ara-C is deoxycytidine deaminase present in the human liver, spleen, plasma and in other tissues (Camiener, 1967). Individual variations in intra- and/or extracellular deaminase activity may be responsible for the observed differences in half-life of Ara-C.

Several methods for the determination of Ara-C in plasma have been published (Borsa et al., 1969; Hanka et al., 1970). Baguley and Falkenhaug (1971, 1975) described a bio-assay based on the inhibition of tritiated thymidine (^{3}HTdR) incorporation into DNA of mouse L-cells and spleen cells, or ascitic L-1210 cells in the presence of Ara-C. Concentrations as low as 0.04 mg/l plasma could be detected.

The plasma disappearance curve of Ara-C after a single intravenous injection is biphasic when plotted on a semilogarithmic scale as is shown by Ho and Frei (1972) after injection of ^{3}HAra-C.

In various treatment schedules of AML a bolus injection of 100 mg Ara-C/m^{2}/12 hours is given. To study interindividual differences

in the second phase of the elimination curve of Ara-C after a therapeutic dose of 100 mg/m^2, it was found necessary to develop a more sensitive assay than those described till now.
In this paper an assay is presented based on the inhibition by Ara-C of ^{3}HTdR incorporation into DNA of rat bone marrow cells. Plasma concentrations as low as 0.003 mg/l can be measured.

3.2 MATERIALS AND METHODS

Preparation of blood samples

Blood samples are taken just before and at different time intervals after a rapid injection of Ara-C in a dose of 100 mg/m^2. Blood samples of 8 ml are collected within 10 seconds in pre-cooled tubes containing 150 IU Heparin and 50 µg Tetrahydrouridine and put on ice immediately. Tetrahydrouridine (THU), added to prevent enzymatic deamination of Ara-C (Camiener, 1967), was kindly provided by Dr Harry B.Wood, Chief Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda.
The blood samples are centrifuged at 0^{o}C (2000xg, 15 min) and the plasma is ultrafiltered by centrifugation at 0^{o}C (250xg, 60 min) through conical ultrafilters (Centriflo F 25, Amicon). The ultrafiltered plasma samples are stored at -70^{o}C.

Preparation of the cell suspension

Wistar rats are sacrificed and bone marrow is obtained from both femurs by expulsion with Eagle's Minimal Essential Medium (MEM Gibco, Cat. No. F 14). The cells are suspended and filtered through a nylon filter (pore size 100µm) to remove tissue particles and cell-clumps. The cell number is counted electronically (Coulter Counter ZF) and the cell suspension is diluted to a density of 2 x 10^6 cells/ml in MEM containing 20 % foetal calf serum (Flow Lab.) buffered with 0.02 M Tris-HCl to pH 7.3 and supplemented with Spinner salts (Gibco F-15) and 2 x 10^{-3} M l-glutamine (Schwarz/Mann).

Determination of Ara-C.

Rat bone marrow cell suspension (0.5 ml, 2 x 10^6 cells/ml) is added to 0.5 ml cooled ultrafiltered plasma containing 0.6 nmol (methyl-^{3}H)-thymidine (^{3}HTdR, 1.66 Ci/mmol, Amersham). The samples are incubated at 37^{o}C in a shaking waterbath for 150 min. After incubation the samples are placed on ice and centrifuged (1000xg, 15 min). The sediment is washed three times with 1 ml Krebs Ringer solution buffered with 35 x 10^{-3} M Tris-HCL to pH 7.3 and containing 0.25 x 10^{-3} M 2-deoxythymidine. To remove all acid soluble radioactivity the cells are washed twice with 1 ml 1M $HClO_4$. The residual $HClO_4$ is removed by treating the precipitate with ice cold 99 % ethanol.
The sediment is dissolved in 0.2 ml Hydroxide of Hyamine (Packard) and quantitatively transferred to counting vials with 4 ml distilled water. After addition of 6 ml Instagel (Packard) the radioactivity is counted in a liquid scintillation counter (LKB 81000)and expressed in desintegration per minute (DPM).

3.3 RESULTS

Aspecific effect of various human plasma samples on ^{3}H-thymidine incorporation.

Experiments were performed to exclude differences in thymidine incorporation into DNA of rat bone marrow cells due to aspecific effects of human plasma. As is shown in fig 3.1 the incorporation of ^{3}HTdR into DNA varies considerably after incubation with different plasma samples This phenomenon was observed in healthy individuals as well as in leukaemic patients. Removal of plasma proteins by ultrafiltration through Centriflo ultrafilters abolished this variability in all samples almost completely and resulted in higher incorporation of ^{3}HTdR.
In control experiments with ^{3}HAra-C, binding to plasma proteins and adsorption to the filter was found to be negligible.

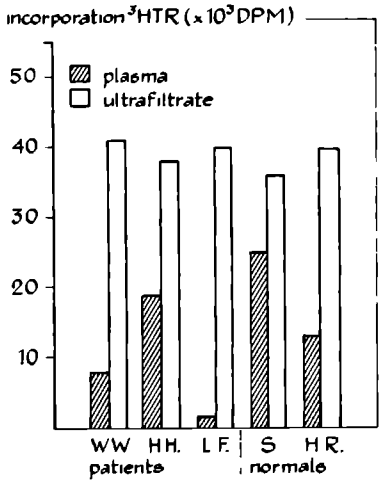

Fig 3.1
Incorporation of ^{3}HTdR into DNA of 10^{6} rat bone marrow cells after incubation for one hour with plasma or ultrafiltered plasma of five different individuals.

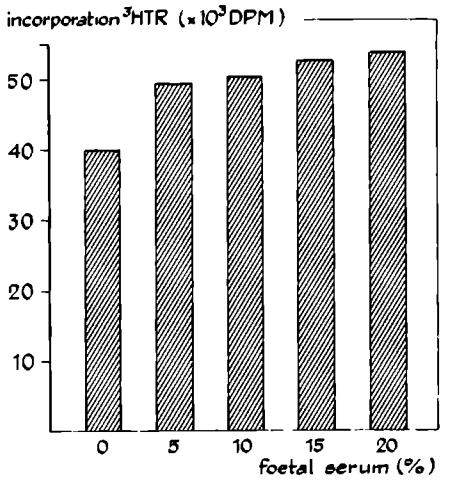

Fig 3.2
Incorporation of ^{3}HTdR into DNA of 10^{6} rat bone marrow cells after incubation for one hour. Ultrafiltered plasma was added to the cell suspension in MEM, supplemented with different amounts of foetal calf serum.

Inhibition of ^{3}H-thymidine incorporation by cytosine arabinoside

The incorporation rate of ^{3}HTdR into DNA of rat bone marrow cells depends strongly on conditions during incubation. It is found that the incorporation per hour increases after addition of different amounts of foetal calf serum. A concentration of 20 % is found optimal causing an increase in incorporation of 30 % compared to the control value (fig 3.2).
In fig 3.3 it is shown that in the abscence of Ara-C a linear relationship exists between the incorporation of ^{3}HTdR into DNA and time. However, after 90-120 min the incorporation rate seems to decline. The same figure demonstrates that the incorporation of ^{3}HTdR is inhibited in the presence of different amounts of

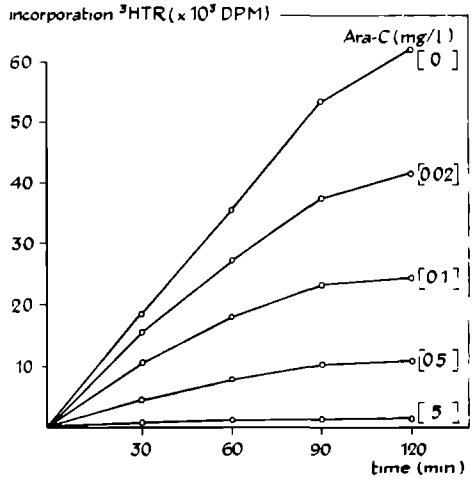

Fig 3.3
Incorporation of ^{3}HTdR into DNA of 10^{6} rat bone marrow cells after different time intervals.
To the cell suspension in MEM an equal amount of ultrafiltered plasma was added, containing different amounts of Ara-C (concentration range 0-5 mg/l).

Ara-C. It can be seen that the incorporation rate of ^{3}HTdR is inhibited proportionally to the Ara-C concentrations. A high concentration of Ara-C (5 mg/l) inhibits the ^{3}HTdR incorporation almost completely within 30 minutes while a low concentration of Ara-C (0.02 mg/l) does not yet inhibit DNA-synthesis completely after two hours of incubation.

The determination of the calibration curve

The inhibition of ^{3}HTdR incorporation in the presence of Ara-C can be expressed as an inhibition percentage (IP);

$$IP = \left[1 - \frac{A}{B}\right] \times 100\ \% \qquad (1)$$

In formula (1) A is the incorporation of ^{3}HTdR in the presence of Ara-C and B the ^{3}HTdR incorporation without Ara-C.

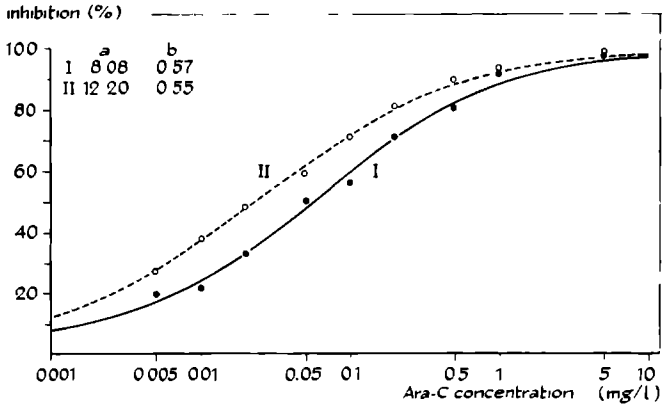

Fig 3.4

The percentages of inhibition of ^{3}HTdR incorporation into DNA of 10^6 rat bone marrow cells is plotted versus the final Ara-C concentrations. The data are obtained with bone marrow from two different rats (o,●) using the same Ara-C solutions. The curves are obtained by calculating parameters a and b from equation (2) in a least squares procedure.

For the determination of unknown concentrations of Ara-C a calibration curve is made by plotting the inhibition percentages versus the logarithm of known Ara-C concentrations. The IP's are measured in triplicate for concentrations $\leq$ 0.05 mg/l and in duplicate for higher concentrations.
The known concentrations are prepared in ultrafiltered plasma samples and their IP's are fitted in a least squares procedure to the equation:

$$IP = 100 \left[1 - \frac{1}{a[\text{Ara-C}]^b} \left(1 - e^{-a\,[\text{Ara-C}]^b} \right) \right] \quad (2)$$

The mathematical derivation and the meaning of parameters a and b are explained in the "appendix". A change in parameter a is related to the horizontal shift of the calibration curve and b is correlated with the slope of the steepest part of the S-curve

Fig 3.4 demonstrates the experimentally determined inhibition percentages plotted versus the known concentrations of Ara-C. It also shows the calculated curve after determination of parameters *a* and *b*. The theoretical curve fits the experimental data rather well which gives an indication for the validity of the model from which equation (2) has been derived (see appendix).

The accuracy of the determination of unknown Ara-C concentrations.

The shape of the calibration curve depends on the sensitivity of rat bone marrow cells for Ara-C. To test this biological variation in sensitivity, five calibration curves were processed with one set of known Ara-C concentrations and the bone marrow of five different rats. The parameters *a* and *b* and their standard deviations calculated for these curves are listed in table 3.I

Rat	*a*	SD *a*	*b*	SD *b*
1	9.5	1.2	0.58	0.04
2	8.1	0.7	0.57	0.03
3	9.8	0.4	0.56	0.01
4	12.2	0.8	0.55	0.02
5	10.0	2.1	0.67	0.08

Table 3.I
Parameters a and b (equation 2) and their standard deviations (SD) calculated from five calibration curves processed with one set of known Ara-C concentrations and the bone marrow of five different rats.

For the analysis in the least squares procedure it was not found necessary to use a weighting scheme for the average IP's of duplicate (high concentrations) and triplicate (concentrations ≤ 0.05) measurements.
The calibration curve processed with the use of bone marrow of rat 2 and 4 are plotted in fig 3.4.

The two curves are significantly different, indicating a variation in sensitivity of the bone marrow cells for Ara-C. Therefore accurate determinations of unknown concentrations will only be obtained if they are processed in the same bone marrow cell suspension as used for the calibration curve.
To get an estimate of the accuracy of the assay we calculated the standard deviation of the logarithm of an Ara-C concentration determined in the plasma of a patient. For this particular calibration curve the parameters a and b are 8.75 (SD 0.56) and 0.52 (SD 0.02) respectively. An inhibition percentage of 50 % (SD 2 %) corresponds here to a concentration of 0.076 mg/l with an error margin of 0.126 and 0.044 mg/l respectively (95 % confidence level). In the experiments unknown concentrations are measured in duplicate which reduces the error margin to 0.110 and 0.052 mg/l respectively. The value 8.75 for parameter a is rather low (table 3.1) and corresponds to a relatively insensitive rat bone marrow.
Since in the outer parts of the S-curve (IP > 80 % and < 20 %) the relative errors will increase, it is necessary to dilute the high concentrations to bring them in the linear part of the calibration curve. Higher values for parameter a do not change the slope of the straight part of the calibration curve. Therefore the relative error will not decrease but lower concentrations can be determined. Parameter b affects the slope of the S-curve. High values for b correspond to steeper curves which therefore reduce the relative errors in the measured concentration.

The sensitivity of the assay

Fig 3.3 shows that the sensitivity of the test increases with the incubation time, which can also be concluded from the mathematical approach (see appendix equation (6)).
However, fig 3.3 also demonstrates that the incorporation of ^{3}HTdR declines after 90 minutes of incubation which limits the increase in sensitivity and also affects the accuracy. After an incubation time of 150 minutes, a compromise between sensitivity and accuracy, the lower limit of the assay proves to be

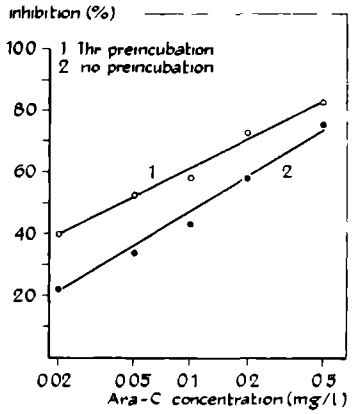

Fig 3.5

The percentages of inhibition of ^{3}HTdR incorporation into DNA of 10^{6} bone marrow cells is plotted versus the Ara-C concentrations. Calibration curve 2 is obtained after incubation of the cell suspension with ^{3}HTdR and Ara-C for two hours. Calibration curve 1 is obtained after preincubation of the cell suspension with Ara-C for one hour followed by another hour of incubation with ^{3}HTdR.

0.003 - 0.008 mg/l depending on the sensitivity of the bone marrow. The incorporation of ^{3}HTdR in the presence of Ara-C decreases as a function of time. Therefore, preincubation with Ara-C in the absence of ^{3}HTdR will increase the sensitivity of the assay. In fig 3.5 it is shown that preincubation with Ara-C for one hour followed by incubation with ^{3}HTdR for another hour indeed increases the sensitivity of the assay considerably.

Assay of cytosine arabinoside in human plasma.

After an intravenous bolus injection of Ara-C in a dosage of 100 mg/m^{2} blood samples were taken every 2 minutes during the first 10 minutes and every 5 minutes during the next 50 minutes. After ultrafiltration the duplicate samples of the unknown concentrations and the samples for the calibration curve are incubated with the same rat bone marrow cell suspension.

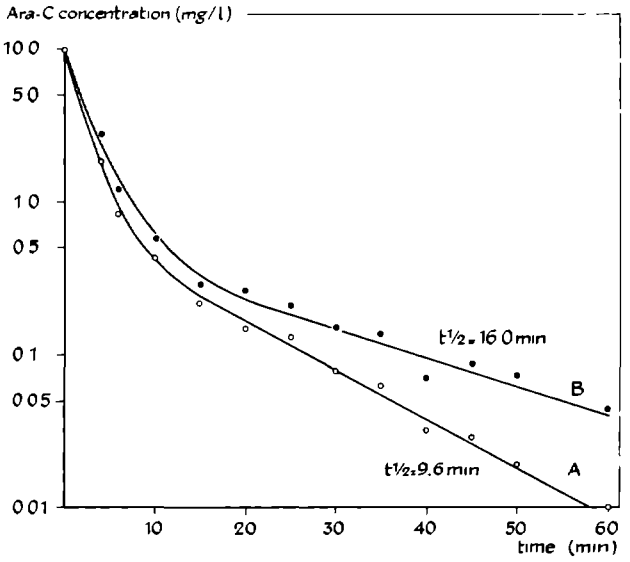

Fig 3.6

Plasma disappearance of Ara-C in two AML-patients after an intravenous push injection (100 mg/m^2). At different times blood was taken and Ara-C determined in the ultrafiltered plasma samples. From these data, half life values were calculated using a two compartment open model.

The samples collected in the first 10 minutes after the injection of Ara-C are diluted three times with ultrafiltered plasma containing no Ara-C to bring the concentration in the linear part of the calibration curve.

Fig 3.6 shows a biphasic elimination pattern of Ara-C from the blood in two AML patients before treatment. The curves are fitted with a least squares procedure* to a two compartment open model to calculate the plasma half-life of Ara-C. The half-life values of the two phases are 1.4 (SD 0.1) and 9.4 min (SD 0.4) for patient A and 1.8 (SD 0.2) and 16.0 min (SD 1.8) for patient B.

*Weighting scheme based on a constant relative error in the measured concentration.

3.4 DISCUSSION

The assay of Ara-C described in this paper does not require tissue culture facilities (Baguley et al., 1971) or tumor transplantation (Baguley et al., 1975) and is more sensitive. The test is based on the inhibition of ^{3}HTdR incorporation in the presence of Ara-C.

It has been reported that the sensitivity of cells to Ara-C correlates with their metabolic activities (Ho et al., 1973). The drug is either phosphorylated to its monophosphate by deoxycytidine kinase (K) (Chu et al., 1965; Momparler et al., 1971) and further phosphorylated to its triphosphate (Ara-CTP) or deaminated to uracil arabinoside by deoxycytidine deaminase (D) (Chabner et al., 1974). The active intracellular form of Ara-C is Ara-CTP causing inhibition of DNA synthesis most likely by inhibition of DNA polymerase (Momparler et al., 1972). The inhibition of DNA synthesis is greater and more pronounced at higher levels of Ara-CTP. Sensitive cells possess high K activities and low D activities (Ho et al., 1972; Furner et al., 1975). We therefore assume that the high sensitivity of rat bone marrow cells to Ara-C is due to a high K/D ratio.

The accuracy of the test is calculated using the mathematical approach of the interaction between the ^{3}HTdR incorporation into DNA and the inhibition of the ^{3}HTdR incorporation by Ara-C which is elaborated in this paper. The lower limit of the assay was 0.003 mg/l plasma.

In general the assay appears to be sufficiently sensitive to determine the plasma disappearance of Ara-C in patients. In the case of a high elimination rate, the assay can be made more sensitive at lower concentrations by a slight modification. It is shown in fig 3.5 that preincubation with Ara-C indeed increases the sensitivity of the assay considerably. However, preincubation requires exact timing of the second incubation and therefore complicates the method considerably, since for the determination of one single disappearance curve about sixty samples have to be processed.

For this reason this modification is restricted to selected cases.
After an intravenous bolus injection of Ara-C the shape of the elimination curve in plasma appears to be biphasic (fig 3.6). The first phase mainly represents the rapid distribution of the drug over the total body water and the second phase reflects the elimination of the drug. Individual differences in the elimination rate correlate with the plasma half-life of the second phase.
In this paper it is shown that a significant interindividual difference may exist in the plasma half-life of Ara-C, after a bolus injection in a dose of 100 mg/m^2.
Dosage schedules for Ara-C related to body weight or body surface, such as used in treatment protocols in acute leukaemia, do not take into consideration large variations in the elimination rate of the drug. In a number of patients treatment with Ara-C must be either ineffective or toxic. It is to be expected that the results of leukaemia treatment will improve when the dosage of Ara-C can be related to optimal plasma levels during therapeutic courses.

APPENDIX

The relation between the inhibition of ^{3}HTdR incorporation and the concentration of Ara-C expressed in equation (2) is derived from a model of two integrated processes. One process represents the incorporation of tritiated thymidine into DNA of rat bone marrow cells (DNA_c) with the incorporation rate constant k_o

$$DNA_c + {}^3HTdR \xrightarrow{k_o} DNA^* \quad \text{(labeled DNA)} \qquad (3)$$

Since ^{3}HTdR is available in excess, (3) can be described as:

$$\frac{d\,(DNA^*)}{dt} = k_1 \cdot DNA_c\,(t) \qquad (3a)$$

where $k_1 = k_o \cdot [{}^3HTdR]$.

The other process represents the inhibition of DNA synthesis by Ara-C with a rate constant k_2

$$DNA_c + \text{Ara-C} \xrightarrow{k_2} DNA_i \text{ (inhibited DNA)} \qquad (4)$$

It can be concluded from fig 3.3 that the inhibition of DNA synthesis in the presence of Ara-C is not linearly proportional to the Ara-C concentration. Therefore a parameter b is introduced to approach this relation;

$$\frac{d\,(DNA_c)}{dt} = -k_2 \cdot [\text{Ara-C}]^b \cdot t \qquad (4a)$$

After integration it follows that the DNA-synthesis declines exponentially in time:

$$DNA_c\,(t) = DNA_o \cdot e^{-k_2\,[\text{Ara-C}]^b \,.\, t} \qquad (5)$$

DNA_o represents the initial number of rat bone marrow cells.

After introduction of (5) in (3a) the rate of labeled DNA synthesis is derived.

$$\frac{d\,(DNA^*)}{dt} = k_1 \cdot DNA_o \cdot e^{-k_2\,[Ara\text{-}C]^b} \cdot t \qquad (6)$$

Equation (6) is integrated over the incubation time and substituted into (1) to give (2).
From (6) it is obvious that the time for complete inhibition is inversely proportional to $[Ara\text{-}C]^b$ which is in good relation with the experimental findings ($b \cong 0.5 - 0.6$)

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CHAPTER 4

PHARMACOKINETICS OF CYTOSINE ARABINOSIDE IN ACUTE MYELOID LEUKAEMIA

ABSTRACT

In fourteen patients with acute myeloid leukaemia (AML) the plasma concentrations of cytosine arabinoside (Ara-C) were determined at the start of the first course of treatment at various intervals after a bolus injection. In ten patients plasma concentration time data were fitted to a biexponential equation and pharmacokinetic parameters were estimated from the coefficients and exponents of such equations. The plasma half-life value of Ara-C of the first phase varied from 1.2 to 1.9 minutes with a mean of 1.6 minutes. The half-life value of the second phase varied from 8.8 to 18.9 minutes.

All patients were treated with Ara-C alone in a dose of 100 mg/m^2 for 10 or 14 days. A poor treatment response was observed in 5 patients with second phase plasma half-life values of Ara-C ranging from 6.6 to 10.7 minutes. A complete remission was attained in 9 patients with half-life values exceeding 12.7 minutes. In three patients plasma Ara-C concentrations were measured during constant rate infusion of different amounts of drug. It appeared that the plateau concentrations were directly proportional to the dose, which indicated that in the therapeutic range no enzyme capacity-limited elimination occurs.

4.1 INTRODUCTION

Cytosine Arabinoside (Ara-C) is one of the most powerful drugs in the treatment of acute myeloid leukaemia (AML). With several treatment protocols a complete remission can be obtained in more than 50 % of the patients (Clarkson, 1972; Mathé, 1976). The drug is transported from the extracellular fluid into the cell by a carrier-mediated diffusion process which increases the rate of uptake (Mulder et al., 1975). Intracellularly the drug is deaminated to uracil arabinoside (Ara-U) or phosphorylated to arabinosyl cytidine triphosphate (Ara-CTP). Only as Ara-CTP is the drug active and competes with deoxyribosylcytidine triphosphate (dCTP) for the binding sites on DNA polymerase, resulting in inhibition of DNA synthesis (Graham et al., 1970). In murine leukaemia it has been demonstrated that the level of Ara-CTP in the cell and the time during which it is present, determine the chemotherapeutic effect of the drug (Chou et al., 1975). The level of Ara-CTP reached in these cells depends in the above-mentioned enzymatic activities and the exposure time of the cells to a presumed minimal effective concentration in the extracellular fluid.

The therapeutic effect of Ara-C alone in the treatment of AML largely depends on the dosage and the mode of administration (Ellison et al., 1968). In a computer model (Mellett, 1972) based on the elimination studies performed by Ho et al. (1971) and continuous infusion studies of Ellison et al.(1968), serum levels of Ara-C were calculated and related to the therapeutic response in adult patients with AML. It was concluded that a minimal effective serum concentration of Ara-C between 0.01 and 0.1 mg/l must be maintained for a sufficiently long period in order to obtain a maximal effect. Differences in the rate of elimination of Ara-C and therefore in blood levels may be the cause of therapeutic failure. The plasma disappearance of Ara-C in man has been determined in several studies, and wide variations exist in the calculated plasma half-life values between the investigators (Creasey et al.,

1966; Ho et al., 1971; Momparler et al., 1972; Baguley et al., 1975).
In this study 14 AML patients were treated with bolus injections of Ara-C. The plasma disappearance curves were determined after the first injection and plasma concentration time data were fitted to a biexponential equation. The pharmacokinetic parameters were estimated from the coefficients and exponents of such equations. The relation between the plasma half-life and the results of remission inducing therapy was examined.

4.2 MATERIALS AND METHODS

Patients

Fourteen AML-patients were studied; six males and eight females. The mean age was 42 (ranging from 20 to 66). The diagnoses were confirmed at the W.H.O. Reference Centre for Leukaemias, Villejuif, France and the Leukaemia Working Group in the Netherlands.

Treatment

Patients were treated with bolus injections of Ara-C at a dose of 100 mg/m^2 every 12 hours. The duration of treatment was adapted to the percentage of DNA-synthesizing cells (S-phase cells) in the bone marrow as measured at the start of therapy by pulse-cytophotometry (Hillen et al., 1975). Patients with less than 10 percent of S-phase cells were treated during 14 days, while those with more than 10 percent of proliferating cells were treated for 10 days.
The bone marrow response after a course of treatment was studied three weeks after the end of Ara-C therapy, when repopulated bone marrow could be expected. Complete remission was defined as a state of normal bone marrow cellularity (less than 5 % blast cells) and normal peripheral blood values. A second course was given if partial remission (5 % < blast cells < 40 %) was

obtained after 10 or 14 days' treatment. In cases of failure (blast cells > 40 %) a second course was given with an increased dose of Ara-C combined with Adriamycin and Thioguanine.
During and after Ara-C treatment patients were hospitalized in germ-poor isolation units and most patients received prophylactic antibiotic medication. All patients received supportive treatment with granulocytes and platelets concentrates during the aplastic phase.

Blood sampling

Eight ml blood samples were obtained by venipuncture, collected in pre-cooled tubes containing 150 IU Heparin and 50 µg Tetrahydrouridine and put on ice immediately. Blood samples were taken after the first bolus injection at the start of therapy, every 2 minutes during the first 10 minutes and every 5 minutes during the next 50 minutes. Tetrahydrouridine, added to prevent enzymatic deamination of Ara-C (Camiener, 1967), was kindly provided by Dr. Harry Wood, Chief Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, U.S.A.
To avoid drug interference during determination of the Ara-C plasma disappearance, all comedication was stopped 12 hours before the drug was administered.

Assay procedure

Blood samples were centrifuged at $0^{o}C$ (2000xg, 15 min). The plasma was ultrafiltered at $0^{o}C$ (250xg, 120 min) through ultrafilters (Centriflo F 25, Amicon) to remove the plasma proteins which disturb the assay (Chapter 3). The fraction of Ara-C bound to plasma proteins was about 13 % (Chapter 5). During filtration the protein bound drug retained in the filter. The free drug appeared in the ultrafiltrate and was measured in the assay. In control experiments with labeled Ara-C, adsorption to the filter was found to be negligible. The Ara-C concentrations in the ultrafiltrate were determined in a bio-assay based on inhibition

of tritiated thymidine incorporation in rat bone marrow cells as described in chapter 3.

Measurement of DNA content

DNA of bone marrow cells binds proportionally the fluorescent dye ethidium bromide. The extent of fluorescence is determined in a cellsuspension by flow-microfluorometry (ICP-II, Phywé, Göttingen, West Germany).
The distribution of the relative DNA content in the cells is plotted as a histogram. The percentage of diploid (2n) G_1-cells, tetraploid (4n) G_2 + M-cells and interploid (2n < DNA < 4n) S-phase cells was calculated from the DNA histogram by planimetry.

Mathematical assessment of experimental determined Ara-C concentrations.

a) Bolus injection of Ara-C

The plot of the logarithmic plasma Ara-C concentrations versus time appeared to be biphasic. This is rather typical for the distribution of drug in a two compartment open model. The plasma concentration in the central compartment can be obtained from the equation:

$$C_1(t) = A.e^{-\alpha t} + B.e^{-\beta t} \qquad (1)$$

A and B are the hybrid constants for the function, α and β are the reciprocal time constants. Preliminary estimates of the parameters of equation (1) were obtained by graphical methods, then data were fitted by non linear least squares regression using a suitable program and an IBM 370 computer. The least squares values of A, α, B, β, were utilized to estimate the pharmacokinetic parameters: V_{dss}, V_1, CL_p, V_{darea}, k_{12}, k_{21}, k_{el}, $(t_{\frac{1}{2}})_\alpha$ and $(t_{\frac{1}{2}})_\beta$ of the two compartment open model with elimination only from the central compartment (Wagner, 1975). For symbols see table 4.I.

b) Constant rate infusion of Ara-C

During constant rate infusion, the drug is distributed over the two compartments and equilibrium is attained when the time of infusion is much longer than the reciprocal time constant β. The increase of drug concentration in the central compartment (C_1) is calculated with equation (2) representing a two compartment open model where elimination occurs only from the central compartment (Wagner, 1975).

$$C_1 = \frac{D}{T.Cl_p}\left[1 - \left(\frac{k_{el} - \beta}{\alpha - \beta}\right) e^{-\alpha t} - \left(\frac{\alpha - k_{el}}{\alpha - \beta}\right) e^{-\beta t}\right] \qquad (2)$$

D is total amount of drug administered
T is the time of infusion

4.3 RESULTS

Bolus injection of Ara-C.

Plasma concentrations of Ara-C after a bolus injection in a dose of 100 mg/m^2 body surface area were measured in 14 AML patients at various intervals as indicated in "methods". The plasma disappearance curve determined in one of the patients is demonstrated in fig 4.1 The shape of the curve appears to be biphasic. Such a pattern is typical for a two compartment open model in which the drug equilibrates from a central compartment into a peripheral one and is only eliminated from the central compartment. The pharmacokinetic parameters of this model are calculated for 10 patients as indicated in "methods" and listed in table 4.I.
The plasma concentration fell rather rapidly during the first phase $(t_{\frac{1}{2}})_\alpha$ with half-life values ranging from 1.2 to 2.0 minutes and more slowly during the second phase $(t_{\frac{1}{2}})_\beta$ with half-life values ranging from 8.8 to 18.9 minutes. The mean rate of elimination of the drug (Cl_p) was 6.29 l/min ranging from 3.84 to 10.08 l/min.

Patient Initials	Age years	Dose mg	Body surface m^2		V_1 l	k_{12} min^{-1}	k_{21} min^{-1}	k_{el} min^{-1}	Cl_p $l\,min^{-1}$	Cl_{p/m^2} $l\,min^{-1}m^{-2}$	V_{dss} l	V_{darea} l	$(t_{\frac{1}{2}})_\alpha$ min	$(t_{\frac{1}{2}})_\beta$ min
L T	29	200	1 67		28 77	0 09	0 05	0 35	10 08	6 04	84 4	272 4[illegible]	1 54	18 91
				SD	4 29	0 02	0 003	0 02	1 38	0 83	18 70	46 67	0 13	1 95
J H	56	200	1 64		24 27	0 16	0 06	0 39	9 48	5 78	93 38	249 47	1 22	18 03
				SD	5 60	0 06	0 01	0 06	1 96	1 20	34 30	66 57	0 16	3 04
J K	45	160	1 62		17 02	0 10	0 06	0 27	4 59	2 83	42 58	102 00	1 81	15 47
				SD	4 36	0 04	0 01	0 04	1 01	0 62	15 00	27 61	0 26	2 44
C A	53	200	2 10		17 79	0 10	0 06	0 36	6 41	3 05	45 39	133 54	1 46	14 56
				SD	3 39	0 04	0 01	0 06	1 35	0 46	16 00	34 35	0 18	2 22
M T	39	160	1 58		16 23	0 08	0 07	0 27	4 31	2 73	34 66	82 00	1 88	13 30
				SD	3 07	0 03	0 01	0 03	0 75	0 47	8 99	17 22	0 20	1 55
J W	46	150	1 58		16 23	0 08	0 80	0 24	3 84	2 43	32 62	71 [illegible]	2 04	12 73
				SD	3 62	0 03	0 01	0 03	0 81	0 51	9 6	16 85	0 [illegible]	1 37
A A	66	185	1 84		18 74	0 13	0 10	0 30	5 65	3 07	43 60	86 92	1 46	10 60
				SD	2 67	0 02	0 01	0 03	0 65	0 35	5 98	95	0 14	0 55
F H	28	170	1 65		17 00	0 11	0 10	0 35	6 02	3 65	35 80	[illegible]	39	9 46
				SD	2 38	0 01	0 01	0 02	0 84	0 21	5 60	2[illegible]	0 12	0 40
P C	20	200	1 81		16 69	0 02	0 08	0 39	6 13	3 39	20 03	6 [illegible]	69	0 48
				SD	3 50	0 02	0 03	0 04	1 48	0 82	6 54	36 93	0 19	3 35
J L	31	170	1 72		16 73	0 06	0 09	0 38	6 43	3 74	16 73	81 39	1 51	8 7[illegible]
				SD	3 64	0 02	0 01	0 04	1 38	0 80	3 64	19 71	0 18	0 98

Table 4 I

Plasma concentration time data of 10 AMI patients were fitted to a biexponential equation to estimate the volume of the central compartment (V_1), the rate constants of drug transport between the first and second compartment (k_{12}, k_{21}) the rate constant of elimination (k_{el}), the elimination clearance constant (Cl_p), the volume of distribution steady state (V_{dss}), the volume of distribution calculated from the area under the curve (V_{darea}) and the half-life values of the first and second phase of the elimination curve $(t_{\frac{1}{2}})_\alpha$ and $(t_{\frac{1}{2}})_\beta$ respectively.

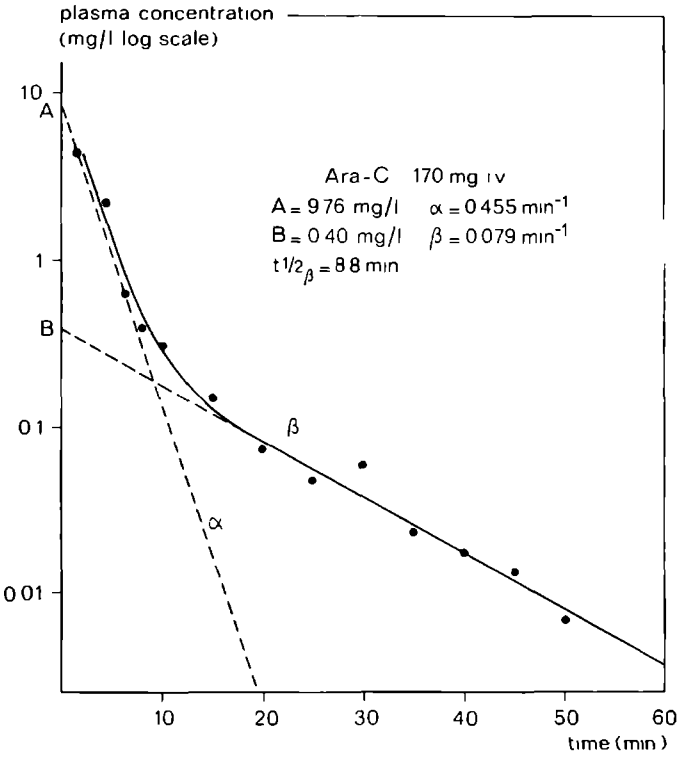

Figure 4.1

Plotted are the plasma Ara-C concentrations versus time after an intravenous bolus injection. The closed circles represent the experimental data and the drawn line the calculated curve (equation 1). The parameters A, B, α and β can be derived from this curve as described in "methods".

Constant rate infusion of Ara-C.

In three patients plasma Ara-C concentrations were measured during constant rate infusion of different amounts of Ara-C. The findings in one of the patients are shown in fig 4.2. It appeared that the plasma Ara-C concentrations measured were directly proportional to the dose. Plateau levels were reached in about 60 minutes with different doses.

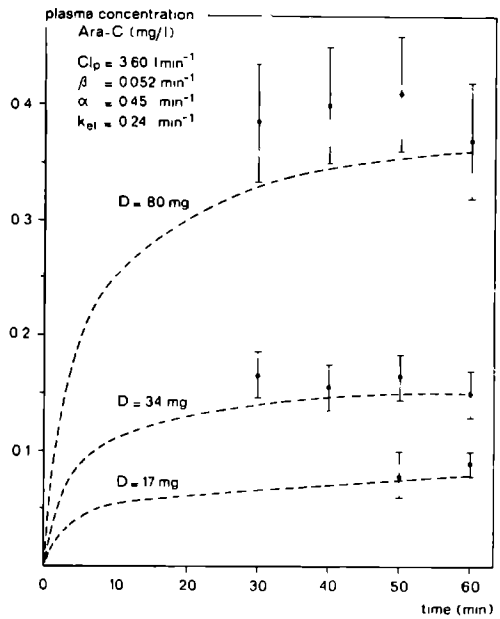

Figure 4.2

Plotted are the plasma Ara-C concentrations versus time during constant rate infusion. The determination of Ara-C in plasma samples was performed in triplicate, and the average Ara-C concentration with its standard deviation is represented by the closed circles and the error bars respectively. The theoretical increase of the plasma Ara-C concentrations during constant rate infusion of a certain amount of drug (D) is calculated as indicated under "methods" and represented by the broken lines.

With use of the pharmacokinetic parameters (Cl_p, k_{el}, α and β) obtained from the elimination curve after a bolus injection, theoretical plasma concentrations during constant rate infusion were calculated (see methods). As is shown in fig 4.2 the experimental and calculated plateau levels were in good agreement.

Plasma Ara-C levels before and at the end of a treatment course.

Remission inducing therapy with Ara-C in AML patients was administered in bolus injections every 12 hours. The drug levels were determined on the first and the tenth day of a treatment course in three patients. As shown in fig 4.3, no differences in drug levels were found after the first and twentieth administration of Ara-C.

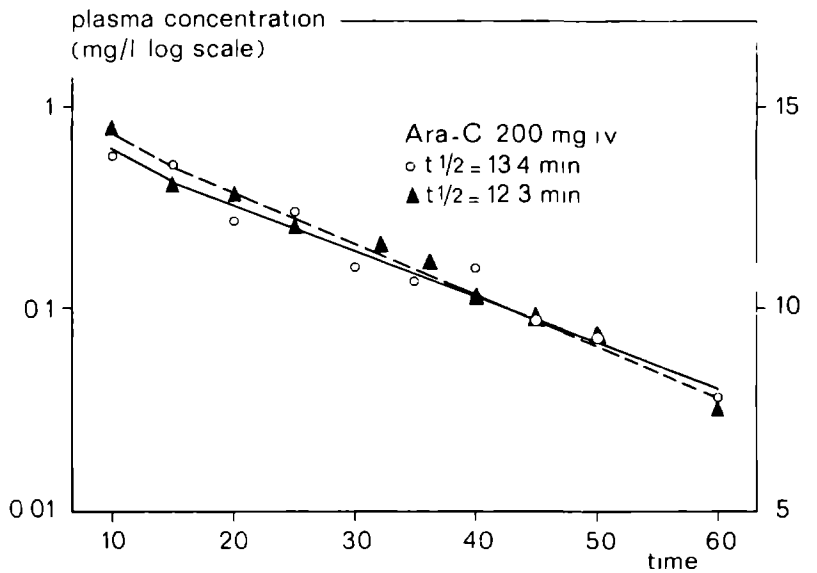

Figure 4.3
Plotted are the experimental and calculated concentrations of Ara-C versus time after the first (o---o) and the 20th (▲-▲) bolus injection during a treatment course in one patient.

Pharmacokinetics of Ara-C in relation to the results of remission inducing therapy

Remission inducing therapy consisted in bolus injections of Ara-C in a dose of 100 mg/m^2 every 12 hours on 10 or 14 consecutive days, depending on the proliferation activity of the bone marrow as measured by pulse cytophotometry (table 4.II). Results of therapy were studied three weeks after the end of a course and were defined as complete remission (CR), partial remission (PR) or failure (F), as indicated under "methods".
In cases of failure a second and third course was given in combination with other drugs. These courses were not included in this study.

Patient	Sex	Age	Diagnosis	State of disease	% S phase cells	Treatment courses (days)	Half life $(t_{\frac{1}{2}})_{\beta}$ (min)	Result of remission induction
L.T.	F	29	AML	Relapse	6	14	18.9	C.R.
J.H.	M	56	AMML	First	13	10 10	18.0	C.R.
H.C.	M	61	AML	First	11	10 6	17.4	C.R.
P.S.	M	41	AML	First	6	14	16.6	C.R.
J.K.	F	43	AML	First	10	10	15.5	C.R.
C.A.	F	53	AML	First	11	14 10	14.6	C.R.
D.S.	F	23	AML	First	9	14	14.3	C.R.
M.T.	F	39	AML	First	13	10	13.3	C.R.
J.W.	F	46	AML	First	N.D.	7 10	12.7	C.R.
A.A.	M	66	AML	Relapse	12	10	10.7	P.R.
H.H.	F	28	AMML	First	13	14	9.5	F
P.C.	M	20	AML	Relapse	12	10	9.5	F
J.L.	F	31	AMML	Relapse	11	10 14	8.8	F
A.K.	M	57	AMML	First	15	10	6.6	F

Table 4.II

In 14 AML patients the relation between the half-life of Ara-C and the result of remission inducing therapy is presented. Complete remission (CR), partial remission (PR) and failure of therapy (F). AML patients are subdivided in acute myeloblastic leukaemia (AML) and acute myelo-monoblastic leukaemia (AMML).

The pharmacokinetic parameters $(t_{\frac{1}{2}})_{\alpha}$, $(t_{\frac{1}{2}})_{\beta}$, Cl_p, V_{dss}, V_{darea}, determined in 14 patients were studied in relation to the results of remission inducing therapy. The first group consisted of nine patients in which complete remission was attained. Five patients in the second group did not come in complete remission (table 4.II). The average of the pharmacokinetic parameters with their range was calculated for each group (table 4.III). Though the number of patients was small, it appeared that the average half-life of the second phase of the elimination curve $(t_{\frac{1}{2}})_{\beta}$ was much longer in the first group compared to the second group of patients. The average values for the elimination clearance constant (Cl_p) and the volumes of distribution (V_{dss} and V_{darea}) did not differ very much between the two groups.

Pharmacokinetic parameters		$(t_{\frac{1}{2}})_{\alpha}$ min	$(t_{\frac{1}{2}})_{\beta}$ min	Cl_p $l\,min^{-1}$	V_{dss} l	V_{darea} l
Group I (n = 9)	$\bar{x}$	1.53	15.14	5.34	40.20	95.41
	range	1.22 - 2.04	12.73 - 18.91	3.84 - 10.08	32.62 - 93.38	71.11 - 272.43
Group II (n = 5)	$\bar{x}$	1.49	9.58	5.99	32.69	85.14
	range	1.39 - 1.73	6.57 - 10.86	3.07 - 5.64	16.73 - 43.60	53.71 - 86.92

Table 4.III

The average of the pharmacokinetic parameters with their range and the result of remission inducing therapy in two groups of patients is studied Group I consists of 9 patients who achieved complete remission and group II consists of 5 patients who achieved partial remission or failure.

For abbreviations of the pharmacokinetic parameters see legend of table 4.I

4.4 DISCUSSION

This study presents pharmacokinetic data on Ara-C in man, derived from the decline of plasma concentrations after a bolus injection. It appears that after the first distribution phase the plasma half-life values of the second phase in 14 AML patients range from 6.6 to 18.9 minutes. These results are in agreement with the data of Momparler et al. (1972) and Baguley et al. (1975). With the aid of tritium-labeled Ara-C, Ho et al. (1971) found a mean of 12 minutes for the first phase and 111 minutes for the second phase. These findings may indicate a third phase in the elimination curve of about two hours. It can be calculated (equation 2) that during constant rate infusion, equilibrium between the two compartments is approximated after at least three times the plasma half-life. Although the data in our infusion experiments (fig 4.2) indicate that equilibrium is attained after about one hour, the infusion time should be at least six hours to show the existence of a third phase in the order of two hours. Thus the possibility of a third phase remains open and in that case the half-life value of the first phase as reported by Ho et al. (1971) corresponds to the half-life values of the second phase in our experiments.

Ara-C is eliminated by glomerular filtration and enzymatic deamination to non-toxic Ara-U (Camiener, 1967). Between 87% and 91 % of the radioactivity recovered in the urine after intravenous administration of labeled Ara-C appeared to be Ara-U (Creasey et al., 1966). To exclude the possibility of an enzyme capacity-limited elimination as reported e.g. for salicylates (Levy et al., 1972) plasma concentrations of Ara-C were measured during constant rate infusion. It appeared that plasma concentrations in the therapeutic range from 0.1 up to 0.4 mg/l were directly proportional to the dose (fig 4.2).

Prolonged administration of this drug might either reduce the plasma half-life as a consequence of increased metabolic elimination, as has been observed for rifampycin (Boman et al., 1972), or increase the blood level in case of accumulation when a long

third phase of at least six hours exists in the plasma disappearance curve (Creasey et al., 1976). In order to exclude these possibilities the level of Ara-C was determined in three patients after the 1st and 20th dose. It appeared that drug level and rate of elimination remained unchanged (fig 4.3).

Since the effect of Ara-C probably depends on the exposure time of blast cells to an effective drug concentration in the extracellular fluid (Chou et al., 1975), we studied the relation between the results of the remission inducing therapy and the pharmacokinetic parameters determined in 14 patients. A complete remission was obtained in 9 patients with average plasma half-life values of 15.1 minutes, and therapy failed in 5 patients with average half-life values of 9.6 minutes (table 4.III). Although the number of patients is small, variations in the rate of elimination or degradation of Ara-C are probably important in predicting the results of chemotherapy. In patients with a short half-life it may be possible to enhance the therapeutic result, either by bolus injections with an increased dose or by continuous infusion to maintain effective drug levels for a sufficiently long time.

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CHAPTER 5

CYTOSINE ARABINOSIDE BINDING TO HUMAN PLASMA PROTEINS

ABSTRACT

The interaction of cytosine arabinoside (Ara-C) with human plasma proteins was investigated by means of ultrafiltration and ultracentrifugation. The results obtained with both methods did not differ significantly. Ara-C binding was studied at plasma levels within the therapeutic range (0.005 - 1.0 mg/l). It appeared that 13.3 % (SD: 2.2 %) of Ara-C in the plasma was bound to proteins. The percentage of bound drug was independent of the drug concentration, at least in the therapeutic range.

5.1 INTRODUCTION

Most drugs circulate in the blood, bound reversibly to plasma proteins. In general, an equilibrium exists between drug molecules free in solution and those bound to proteins. The unbound fraction of the drug is available for transport to the tissues and thus for therapeutic action (Davis, 1943). The bound drug is considered to be a reservoir. While the free drug is being eliminated, the bound drug dissociates from its binding sites and replenishes the supply of free drug. Variations in the extent of binding may result in significant changes in pharmacokinetic and pharmacodynamic behaviour of drugs that are highly bound to plasma proteins (Anton, 1973; O'Reilly, 1973).
Pharmacokinetic studies of the cytostatic drug cytosine arabinoside (Ara-C) in acute myeloid leukaemia (AML) showed the existence of significant variations in the elimination rate in different patients (chapter 3). To investigate the cause of these differences we determined the fraction of Ara-C bound to plasma proteins at therapeutic drug concentrations. Since protein content and composition in the plasma of AML patients are normal, when determined by means of conventional assay methods, this paper reports the results of ultrafiltration and ultracentrifugation studies in plasma samples of healthy volunteers.

5.2 MATERIALS AND METHODS

Tritiated cytosine arabinoside (5-^{3}H Ara-C, 15 Ci/mmol, Radiochemical Centre, Amersham, England) more than 98 % pure, was used. The purity was confirmed by ascending thin-layer chromotography on silicagel glass plates (F257, Merck) using water saturated butanol as solvent (Scheit, 1967).
The determination of the percentage of bound Ara-C was carried out with citrated plasma obtained from healthy volunteers. The drug was dissolved in therapeutic concentrations ranging from 0.005 to 1.0 mg/l plasma. Trace quantities of ^{3}H Ara-C (0.04 µCi/ml plasma) were added. Triplicate samples of 50 µl were

retained for the determination of total radioactivity. The remainder was used in ultrafiltration and ultracentrifugation studies.

Ultrafiltration

Plasma samples of 4 ml were ultrafiltered by centrifugation at 0°C, 250 x g, through conical ultrafilters (Centriflo, F 25, Amicon) as described by Hooper et al.(1975). The experiments were performed at 0°C in order to prevent enzymatic conversion of the drug. From the first ultrafiltrate (approximately 0,5 ml) triplicate samples of 50 µl were taken. The 50 µl samples of plasma and ultrafiltrate were added to 10 ml scintillation fluid (Instagel, Packard) and were counted in a liquid scintillation counter (LKB 81000). The fraction of drug bound to plasma proteins was calculated from the radioactivity in the ultrafiltrate and in the original plasma samples.
Since the amount of protein in the plasma samples varied, a correction was made for the volume displaced by the proteins according to McLean et al (1935). The radioactivity in all plasma samples was multiplied with a factor $100/P_w$ where

$$P_w = 99.6 - (0.75 \times \% \text{ protein}) \text{ ml}/100 \text{ ml plasma.}$$

Ultracentrifugation

Ultracentrifugation of plasma samples was performed in an IEC B-60 ultracentrifuge equiped with a swinging-out rotor (no SB 283) carrying 6 polyaliumer tubes (no 2842, contents 12 ml). Runs were made at 40,000 RPM (30,000 x g), 0°C for 16 hours according to Scholtan (1965). After centrifugation the content of each tube was collected in separate fractions from the top to the bottom. At the top 5 fractions of 0.2 ml were taken followed by 4 fractions of 0.5 ml and 9 of 1.0 ml. When runs were made at 30,000 x g, 0°C for 40 hours, fractions of 0.5 ml were taken. From each fraction triplicate samples of 50 µl were counted for

radioactivity. The percentage of bound Ara-C was calculated after correction for protein volume as described under ultrafiltration. In a separate tube, containing plasma without Ara-C, the same fractions were taken after centrifugation. From each fraction and the original plasma, duplicate samples of 0.1 ml were analysed for protein content.

Determination of protein

Total protein concentrations were determined with the biuret method. The concentration of albumin in the plasma samples was determined with the bromcresol-green method according to Doumas et al. (1971). The globulin content was calculated by subtracting albumin content from total protein.

5.3 RESULTS

Ultrafiltration

The fraction of Ara-C bound to plasma proteins is determined by ultrafiltration taking advantage of the fact that all bound Ara-C is retained in the filter and all free Ara-C will appear in the ultrafiltrate. The extent of protein binding is calculated from the concentration of free drug in the ultrafiltrate and the concentration of total drug in the original plasma sample after correction for the volume displaced by the proteins as described in "methods".
Different Ara-C concentrations in the therapeutic range (0.005-1.0 mg/l) with trace amounts of ^{3}H Ara-C were added to plasma samples of four individuals and were ultrafiltered to determine the extent of protein binding. The results of these experiments are shown in table 5.I. The percentage of drug retained in the filter and probably bound to plasma proteins varies from 12.5 % to 14.0 % and appears to be independent of the drug concentration in the range studied. This assumed protein binding might be caused by adsorption of the drug to the filter material. Therefore, experiments were performed to exclude this possibility.

Ara-C concentration (mg/l)	Ara-C bound (%)	S.D.
1	12.5	2.5 (4)
0.1	12.5	2.1 (4)
0.5	13.8	2.2 (4)
0.05	14.0	2.2 (4)
0.005	13.8	2.1 (4)

Table 5.I

The average percentage of drug bound to plasma proteins with standard deviation (SD) in plasma samples obtained from 4 individuals (n=4) is demonstrated at different Ara-C concentrations. Binding studies were performed by ultrafiltration.

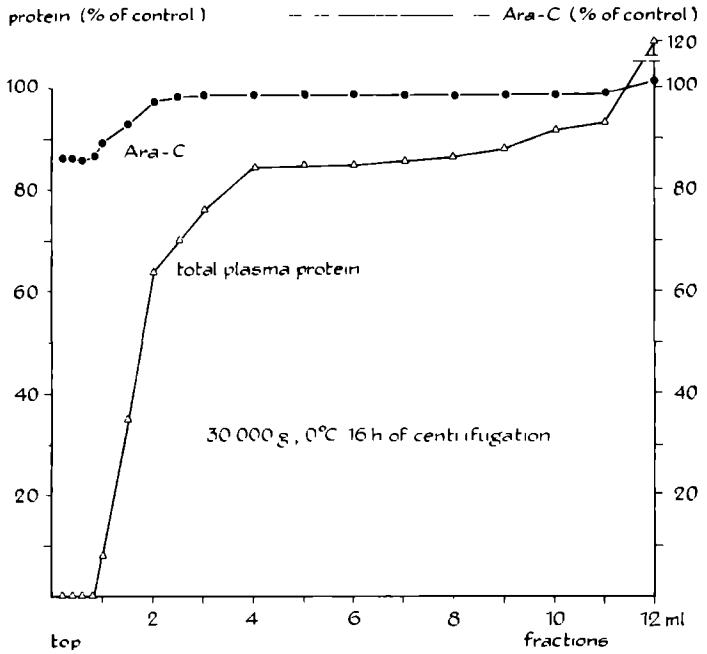

Figure 5.1

Distribution pattern of Ara-C and protein in the centrifuge tube after 16 hours of centrifugation at 30,000 x g, 0°C.

Ara-C (0.05 mg/l) and trace amounts of ^{3}H Ara-C were prepared in plasma ultrafiltrate and filtered again. No significant differences in the radioactivity of the ultrafiltrate before and after the second filtration were found, indicating a negligible adsorption of the drug to the filter.

Ultracentrifugation

The fraction of Ara-C bound to plasma proteins is also determined by ultracentrifugation. In these experiments the extent of protein binding is calculated from the concentration of free drug in the protein free fraction at the top of the centrifuge tube and the concentration of total drug in the original plasma sample. Trace amounts of ^{3}H Ara-C and Ara-C concentrations varying from 0.005 to 1.0 mg/l were added to plasma samples and centrifuged for 16 hours ar 30,000 x g, $0^{o}C$. After centrifugation, fractions were taken and assayed for radioactivity and protein as described in "methods". The distribution of labeled Ara-C and protein in the centrifuge tube is demonstrated in a representative experiment in fig 5.1 A proteinfree supernatant of 0.75 ml and several fractions at which protein and drug are in equilibrium, have

Ara-C concentration (mg/ml)	Ara-C bound (%)	S.D.
1	12.6	2.6 (4)
0.1	13.4	3.5 (4)
0.5	14.1	2.3 (4)
0.05	13.5	3.2 (4)
0.005	12.8	2.9 (4)

Table 5.II

The average percentage of drug bound to plasma proteins with standard deviation (SD) in plasma samples obtained from 4 individuals (n=4) is demonstrated at different Ara-C concentrations. Binding studies were performed by ultracentrifugation at 30,000 x g, $0^{o}C$, for 16 hours.

been found. These findings are in accordance with the data of Scholtan (1965) who studied protein binding of sulfonamides. The percentage of drug bound to protein in the plasma of four individuals varies from 12.6 % to 14.1 % and is independent of the drug concentration in the therapeutic range (table 5.II). Büttner et al. (1961) reported an increase in the concentration of unbound drug in the protein free supernatant after long lasting centrifugation times. Fig 5.2 represents the distribution of drug and protein in the centrifuge tube after 40 hours of centrifugation.
The protein free supernatant is increased (3.5 ml). The protein concentration at the plateau level is decreased by 35 %. However, the percentage of drug in the proteinfree supernatant has not changed.

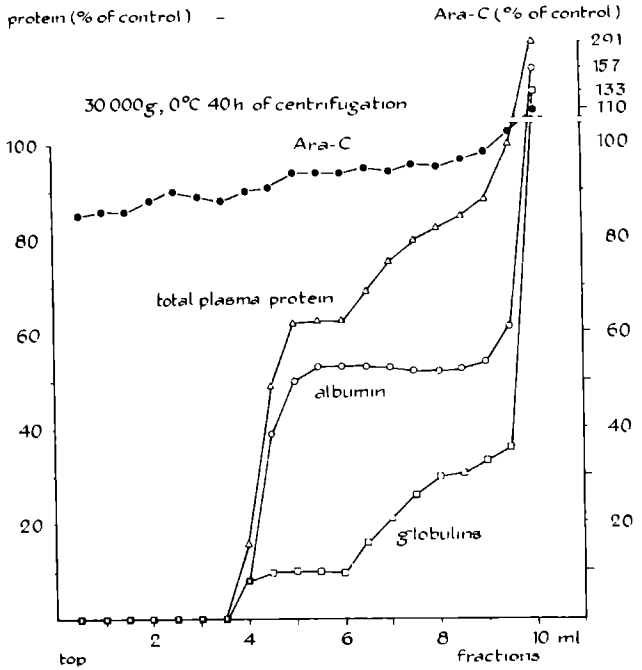

Figure 5.2
Distribution pattern of Ara-C and protein in the centrifuge tube after 40 hours of centrifugation at 30,000 x g, 0°C.
The protein containing fractions were analysed for albumin and globulins.

5.4 DISCUSSION

The percentage of Ara-C bound to plasma proteins has been determined by ultrafiltration and ultracentrifugation.
The principal advantages of ultrafiltration above ultracentrifugation are the economy of time and the ease of performance.
The disadvantage of ultrafiltration may be the adsorption to the filter material. Moreover, an enhanced binding of the drug to plasma proteins in the filter seems reasonable due to increase of protein concentration during filtration (Olsen, 1975).
Therefore protein binding was also studied by ultracentrifugation in which both phenomena are absent. The results obtained with both methods are equal. The percent of Ara-C bound to plasma proteins amounts approximately 13 %.
Scholtan stated that during ultracentrifugation in a swinging-out-bucket rotor the synchronous sedimentation of protein and protein-drug complex as well as the existance of a level where all components are in equilibrium has to be found. The concentration of drug and protein at the plateau level should be equal to the original concentration. In our experiments concentrations of drug and protein at the plateau level were somewhat lower compared with the original concentrations after 16 hours of centrifugation (fig 5.1). This finding was more pronounced after 40 hours of centrifugation (fig 5.2).
The sedimentation rate of the different plasma proteins depends e.g. on the molecular weight which differs markedly for albumin and globulins. After 40 hours of ultracentrifugation the plateau level for proteins had almost vanished and determination of the protein containing fraction for albumin and globulins revealed a variation in the sedimentation rate of the different globulins (fig 5.2). The importance of proteinbinding in pharmacokinetics was recently studied for antipyrine, a drug with a protein binding comparable with Ara-C (Gugler et al., 1975). No significant changes in distribution and elimination of antipyrine were found in patients with normal and very low plasma albumin concentrations. We therefore may conclude that proteinbinding is not a significant factor in the pharmacokinetics of Ara-C.

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CHAPTER 6

THE RELATION BETWEEN THE ARA-C SENSITIVITY AND THE RESPONSE TO TREATMENT IN PATIENTS WITH ACUTE MYELOID LEUKAEMIA

ABSTRACT

A test is presented to predict the response of patients with acute myeloid leukaemia (AML) to cytosine arabinoside (Ara-C). This Ara-C sensitivity test is based on the in-vitro incorporation of tritiated thymidine (^{3}HTdR) into leukaemic bone marrow cells taken *before and one hour after* an intravenous bolus injection of Ara-C.

The decrease of the ^{3}HTdR incorporation after Ara-C administration, expressed as an inhibition percentage, was determined in 11 AML patients and related to the result of remission inducing therapy. An inhibition percentage of more than 70 % was found in seven patients. All these patients came into complete remission after one or two courses with Ara-C alone.

A relatively low inhibition percentage was found in four patients. Three of these patients failed on therapy after two courses and one patient died during the second course. Obviously Ara-C is not the most adequate cytotoxic drug for these patients.

6.1 INTRODUCTION

Cytosine arabinoside (Ara-C) is an analogue of the naturally occurring nucleoside cytidine. The drug is widely used in the treatment of acute myeloid leukaemia (AML) often in combination with other drugs. Unfortunately only in about 60 % of the patients complete remission is attained (Clarkson, 1972; Crowther et al., 1973; Mathé et al., 1976).
After intravenous administration the drug passes the cell membrane and is either phosphorylated or deaminated (Chu et al., 1965; Camiener, 1967). Only in the form of Ara-CTP is the drug active. Presumably it inhibits DNA polymerase by competing with deoxycytidine triphosphate for the binding sites on the enzyme (Graham et al., 1970). The intracellular amount of Ara-CTP is related to the duration of an effective drug level (Mellett, 1972) and to the net effect of the phosphorylating and deaminating enzymatic activities (Ho, 1973).
In this paper an Ara-C sensitivity test is described based on the difference in ^{3}HTdR incorporation into leukaemic bone marrow cells taken before and one hour after a bolus injection of Ara-C. The inhibition of ^{3}HTdR incorporation after Ara-C administration was measured in eleven AML patients and related to the results of remission inducing therapy.

6.2 MATERIALS AND METHODS

Patients

Eleven AML patients were studied; three males and eight females. The mean age was 38 years ranging from 19 to 57 years. Eight patients were first treated and three patients were treated for a relapse. The diagnoses were confirmed at the W.H.O.Reference Centre for Leukaemias, Villejuif, France and the Leukaemia Working Group in the Netherlands.

Treatment

All patients were treated with bolus injections of Ara-C in a dose of 100 mg/m^2 every 12 hours during the first remission inducing course. This course was given for 10 - 14 days. The result of the first course was evaluated at the moment that the bone marrow was repopulated. Complete remission (CR) was defined as a state of normal bone marrow cellularity with less than 5 % of myeloblasts and an adequate peripheral circulatory count of polymorphonuclear leukocytes and platelets. When the number of leukaemic cells in the bone marrow was between 5 % and 40 % the result of treatment was defined as partial remission (PR). No response to therapy in the bone marrow (more than 40 % leukaemic cells) was defined as failure (F).
A second course was given when the first course resulted in a PR or F. In cases of a PR patients were treated with Ara-C alone. In cases of F patients were treated with Ara-C in combination with Adriamycin and Vincristine. In these patients the result of treatment was once more evaluated after the second course. During treatment patients were hospitalized in germ-poor isolation units. All patients received supportive treatment with granulocytes and platelets concentrates during the aplastic phase.

Differences in DNA content of bone marrow cells

DNA of bone marrow cells binds proportionally the fluorescent dye ethidium bromide. The extent of fluorescence is determined in a cell suspension by flow-microfluorometry (ICP-11, Phywé, Gottingen, West Germany).
The distribution of the relative DNA content in a large number of cells is plotted as a histogram. The percentage of diploid (2n) G_1-cells, tetraploid (4n) G_2 + M-cells and interploid (2n < DNA < 4n) S-phase cells was calculated from the DNA histogram by planimetry (Gohde, 1973)

The Ara-C sensitivity test

The test is based on the in-vitro incorporation of ^{3}HTdR into leukaemic bone marrow cells taken before and one hour after an intravenous bolus injection of Ara-C in a dose of 100 mg/m^{2}. These marrow aspirates were taken from patients with a full blown leukaemia at the start of therapy. The cells were diluted in 2 ml of buffered ACD solution (acid citrate dextrose, pH 7.4, formula A, USP XV) and filtered through a nylon filter (pore size 100 µm) to remove tissue particles and cell clumps. The number of nucleated cells was counted electronically (Coulter Counter Z.F.) and adjusted to 10^{6} cells/ml with a solution of Krebs Ringer Tris (pH 7.4) containing 5 % heat-inactivated (56^{o}C) Foetal Calf Serum (KRT-5 % FCS). Twenty ml of this cell suspension was layered upon 10 ml Ficoll Isopaque containing 5 % FCS (spec. grav. 1.074 g/ml at 25^{o}C) according to Loos et al. (1974). After centrifugation (30 min, 1100 x g, 0^{o}C) the cells at the interface were collected by aspiration with a needle punched through the wall of the tube just below the interface. The cells (> 90% blast cells) were washed once with a solution of KRT-5 % FCS. After centrifugation (10 min, 700 x g, 0^{o}C) the cells were resuspended in 1 ml KRT solution 20 % AB serum and diluted to a density of 0,5 x 10^{6} cells/ml. Duplicate samples of 1 ml were incubated with 0.2 nmol tritiated thymidine (^{3}HTdR, spec. act. 5 Ci/mmol) at 37^{o}C for one hour in a shaking water bath. After incubation the samples were placed on ice to stop incorporation and centrifuged (1000 x g, 15 min, 0^{o}C). The pellet was washed three times with 1 ml KRT containing 0.25 x 10^{-3} M 2-deoxythymidine. To remove all acid soluble radioactivity the cells were washed twice with 1 ml 1M $HClO_4$. The residual $HClO_4$ was removed by treating the precipitate with ice cold 99 % ethanol. The sediment was dissolved in 0.2 ml hydroxide of hyamine (Packard) and quantitatively transferred to counting vials with 4 ml distilled water. After addition of 6 ml Instagel (Packard) the radioactivity was counted in a liquid scintillation counter (LKB 81000). The difference in ^{3}HTdR incorporation into the blast cells before and after administration of

Ara-C was expressed as an *inhibition percentage*. The test is summarized schematically in fig 6.1.

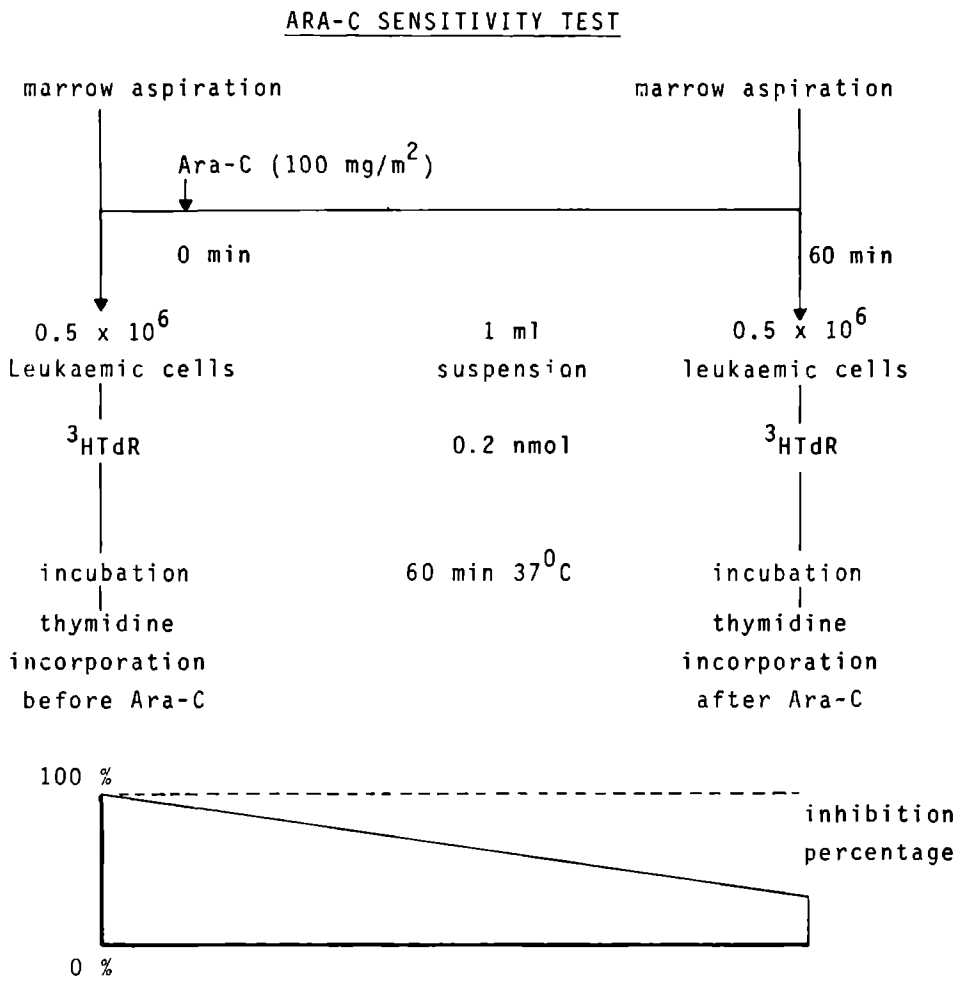

Figure 6.1

Schematic representations of the Ara-C sensitivity test. Leukaemic bone marrow cells, isolated by density centrifugation from marrow aspirates taken before and 60 minutes after Ara-C administration, were incubated with ^{3}HTdR (37°C, 60 min). The difference in the amount of incorporated thymidine is expressed as an inhibition percentage. The details are given in "Methods".

Reliability of the Ara-C sensitivity test

a) Recovery of leukaemic cells.

Since erythrocytes disturb the Ara-C sensitivity test they were removed from bone marrow aspirates by centrifugation on a Ficoll Isopaque cushion as described before. Although almost complete elimination of erythrocytes was achieved, some variable loss of leukaemic cells was unpreventable.
In 5 patients the recovery and the possibility of a selective loss of proliferating cells was studied. Table 6.I shows the recovery of leukaemic cells at the interface from marrow aspirates taken before and after Ara-C The recoveries are almost equal in each individual patient. However, the recovery between the different patients varies This may be due to differences in specific gravity of the leukaemic cells (Zipursky et al., 1976). The individual differences in loss of leukaemic cells could involve preferential loss of proliferating cells. For this reason the percentage of S-phase cells (2n < DNA < 4n) was determined by a flow-microfluorometer. In each patient the percentage of S-phase cells in the bone marrow aspirate taken before and after Ara-C appeared to be almost equal. Also after density centrifugation no significant differences in the percentages of S-phase cells in the isolated leukaemic cells were found in individual patients. In patients with a rather low recovery, the percentage of S-phase cells in the suspension appeared to be increased after Ficoll Isopaque separation. This suggests that non-proliferating cells are preferentially lost during separation.

b) Reproduceability of ^{3}HTdR incorporation

The reproduceability of ^{3}HTdR incorporation was studied in three bone marrow aspirates. Each bone marrow aspirate was divided

into four samples and separated on Ficoll Isopaque. After centrifugation the leukaemic cells were isolated and incubated with ^{3}HTdR. The average variation in the extent of ^{3}HTdR incorporation was 4 % in the four cell suspensions obtained from each bone marrow aspirate.

Patient	Moment of bone marrow aspiration	Recovery of leukaemic cells at the interface	Percentage of S-phase cells	
			Bone marrow aspirate	Isolated leukaemic cells
1	BEFORE ARA-C	80 %	20 %	20 %
	AFTER ARA-C	75 %	18 %	19 %
2	BEFORE ARA-C	67 %	6 %	8 %
	AFTER ARA-C	56 %	6 %	9 %
3	BEFORE ARA-C	75 %	13 %	13 %
	AFTER ARA-C	75 %	12 %	14 %
4	BEFORE ARA-C	89 %	10 %	12 %
	AFTER ARA-C	89 %	11 %	12 %
5	BEFORE ARA-C	58 %	5 %	8 %
	AFTER ARA-C	58 %	5 %	8 %

Table 6.I

The recovery of leukaemic cells and the percentage of proliferating cells are given after density centrifugation of bone marrow samples taken before and 60 minutes after Ara-C administration.

Patient Initials	Age	Sex	Diagnosis	State of disease	Inhibition percentage	Duration first course	Result of therapy	Duration second course	Result of therapy
L.T.	30	F	AML	Relapse	95	14	CR		
J.K.	43	F	AML	First	90	10	CR		
P.S.	41	M	AML	First	86	14	CR		
M.T.	38	F	AML	First	81	10	CR		
C.A.	53	F	AML	First	79	14	PR	10	CR
D.S.	23	F	AML	First	78	14	CR		
J.H.	56	M	AMML	First	73	10	PR	10	CR
S.P.*	19	F	AML	First	69	14	F	6	†
H.H.*	28	F	AMML	Relapse	65	14	F	10	F
J.L.*	31	F	AMML	Relapse	50	10	F	14	F
A.K.*	57	M	AML	First	33	10	F	6	PR

Table 6.II .

The relation between the inhibition of ^{3}HTdR incorporation (inhibition percentage) and the result of Ara-C therapy is demonstrated in eleven AML patients. The patients were subdivided in acute myeloid leukaemia (AML) and acute myelomonoblastic leukaemia (AMML).

During the first and second course patients in general were treated with Ara-C in a dose of 100 mg/m^2 every 12 hours for 10 or 14 days.

* *These four patients were treated during the second course with Ara-C starting on day 3 in combination with Adriamycin (day 1) and Vincristine (day 2). One patient died during treatment (†) CR is complete remission; PR is partial remission; F is failure. (see "methods").*

Relation between the Ara-C sensitivity test and the result of therapy.

The inhibition of ^{3}HTdR incorporation found with the Ara-C sensitivity test ranged from 33 % - 95 % in eleven AML patients (Table 6.II). Four patients with inhibition percentage above 80 % came into complete remission after the first course of treatment.
Three patients had an inhibition percentage within the range of 70 % - 80 %. One of these patients came into complete remission and two patients into partial remission after the first course. These two patients came into complete remission after a second course.
Four patients with inhibition percentages ranging from 33 % to 69 % failed on therapy after the first course. In a second course these patients were treated with Ara-C in combination with Adriamycin and Vincristine. Two patients failed on this second course, one patient came into partial remission and one patient died during treatment.

6.4 DISCUSSION

After intravenous administration of Ara-C the drug is distributed over the entire body and transported into the cell by a carrier mediated diffusion process (Mulder et al., 1975). The amount of Ara-C in the cell depends on the duration of an effective drug level in the blood (Mellet, 1972). In the cell, the Ara-C molecule is either activated by phosphorylation to Ara-CTP (Chu et al., 1965) or inactivated by deamination to Ara-U (Camiener, 1967). Thus, the amount of Ara-CTP formed intracellularly will depend on a) the amount of drug transported into the cell and b) the relative proportions of drug phosphorylated and deaminated. Ara-CTP inhibits DNA synthesis. It is assumed that the inhibition of ^{3}HTdR incorporation reflects the amount of Ara-CTP in the cell.
The test presented in this paper is easy to perform and takes about four hours. The relation between this test and the result

of therapy was studied in eleven patients. An inhibition percentage higher than 70 % was found in seven patients. All these patients came into complete remission after one or two courses with Ara-C alone. Four patients with an inhibition percentage lower than 70 % failed on the first course and were treated during a second course with a combination of Ara-C, Adriamycin and Vincristine. Of the patients treated in this way, only one came into partial remission, one died while under treatment and two failed on therapy (table 6.II).
Although the number of patients is rather small the data suggest that the Ara-C sensitivity test may have a predictive value. Patients with a high inhibition percentage can be treated successfully with Ara-C alone. Apparently patients with a low inhibition percentage are rather insensitive to Ara-C and should not be treated with this drug alone. The test appears to be of lesser value in predicting the response of therapy in patients with inhibition percentages of about 60 % to 70 %.
The predictive value of the test may improve by determining the inhibition of ^{3}HTdR incorporation at other time intervals after Ara-C administration.

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CHAPTER 7

THE ARA-C SENSITIVITY AS A RESULTANT OF PLASMA HALF-LIFE AND INTRACELLULAR DEAMINATING ACTIVITY

ABSTRACT

The amount of tritiated thymidine (^{3}HTdR) incorporated into leukaemic bone marrow cells, obtained from marrow aspirates taken before and one hour after administration of Ara-C, is determined in the Ara-C sensitivity test. The observed decrease of ^{3}HTdR incorporation is expressed as an *inhibition percentage* (I.P.) reflecting the responsiveness to the drug, which will depend on the plasma half-life and the intracellular deaminating activity. To obtain information about the value of the Ara-C sensitivity test, the inhibition percentage of ^{3}HTdR incorporation was measured simultaneously with the plasma half-life and intracellular deamination of Ara-C in 12 AML patients.

Complete remission was achieved in seven patients with an inhibition percentage of more than 70 % and plasma half-life values exceeding 13 minutes. Five of these patients who had low deaminating activities came into complete remission after one course while the two other patients with high deaminating activities needed two courses to achieve complete remission.

A poor bone marrow response was found in three patients with an inhibition percentage below 70 % and plasma half-life values less than 10 minutes. One of these patients with a low deaminating activity came into partial remission after two courses. The other two patients with high deaminating activities failed on prolonged treatment. Two patients died during therapy.

7.1 INTRODUCTION

As described in chapter 4 we found a relation between the plasma half-life of Ara-C and the result of remission inducing therapy. Complete remission was attained in patients with an average plasma half-life of 15 minutes, while patients with an average half-life of 9 minutes showed a poor response to therapy. These data suggest that the plasma half-life determines the amount of Ara-C taken up by the cells.
In the cell the drug is activated by phosphorylation to its triphosphate Ara-CTP (Chu et al., 1965) or inactivated by deamination to uracil arabinoside (Camiener, 1967). Steuart and Burke (1971) observed that the deaminating activity in the leukaemic cells influences the cytotoxic effect of the drug. The Ara-C sensitivity test, described in chapter 6, reflects the net effect of plasma half-life, the deaminating activity as well as the phosphorylating capacity in the leukaemic cells.
In this chapter the value of the Ara-C sensitivity test is studied in relation to the plasma half-life, the deaminating activity and the response to therapy in 12 AML patients.

7.2 MATERIAL AND METHODS

Patients

Twelve AML patients were studied, four males and eight females. The mean age was 39 years (ranging from 19 to 57). Nine patients were first treated and three patients were treated for a relapse. The diagnoses were confirmed at the W.H.O. Reference Centre for Leukaemias, Villejuif, France and the Leukaemia Working Group in the Netherlands.

Treatment

All patients were treated with bolus injections of Ara-C in a dose of 100 mg/m^2 every 12 hours during the first remission

inducing course of treatment. The duration and composition of the second course depended on the result of the first course which was studied when the bone marrow was repopulated. Complete remission was defined as a sta e of normal bone marrow cellularity (less than 5 % blast cells) and normal peripheral blood values. When a considerable decrease in the leukaemic cells of the bone marrow (blast cells between 5 % and 40 %) was found, the result of treatment was defined as a partial remission (PR) and treatment was continued with Ara-C. When no response in the bone marrow (leukaemic cells more than 40 %) was found the result of treatment was defined as a failure (F) and a second course of treatment was given with Ara-C in combination with Adriamycin and Vincristine. The final result of treatment was evaluated after the second course of treatment.
During treatment patients were hospitalized in germ-poor isolation units. All patients received supportive treatment with granulocytes and platelets concentrates during the aplastic phase.

Half-life of Ara-C

The plasma Ara-C concentrations were determined after an intravenous bolus injection of 100 mg/m^2 in a bio-assay as described in chapter 3.

The Ara-C sensitivity test

The estimation of the difference in ^{3}HTdR incorporation in the leukaemic bone marrow cells taken before and 60 minutes after an intravenous bolus injection of Ara-C is described in chapter 6.

Determination of the deaminating activity in leukaemic cells

The ability of the leukaemic cells to deaminate Ara-C was determined in a crude extract of these cells. A marrow aspirate, taken before administration of Ara-C, was layered on Ficoll Isopaque (sg. 1.074 g/ml, 25^{o}C) according to Loos and Roos (1974).

After centrifugation (30 min, 1100 x g, 0°C), the cells at the interface were suspended in Krebs Ringer Tris solution (pH 7.4) to a density of 14 x 10^6 cells/ml. The cells were disrupted by freezing and thawing (5 times). The crude extract (350 µl) was incubated with 100 nmol Ara-C and 0.06 nmol ^{3}H Ara-C (spec. act. 15 Ci/mmol Radiochemical Centre, Amersham) at 37°C for 60 minutes in a total volume of 400 µl. The reaction was stopped by adding 50 µg tetrahydrouridine (20 µl) at 0°C together with an excess of unlabeled Ara-C and Ara-U. A 10 µl aliquot of the reaction mixture was spotted on silicagel glass plates (F 254, Merck, Darmstadt). The plates were developed in water saturated 1-butanol according to Scheit (1967). The Ara-U spots were scraped off and suspended in 4 ml distilled water and 6 ml Instagel (Packard) and assayed for radioactivity in a liquid scintillation counter (LKB 81000). The deaminating activity is expressed in nmol Ara-C/hour/10^8 cells. The enzymatic conversion of Ara-C in Ara-U was linear during 60 minutes of incubation. In cases of a high deaminating activity the crude extract was diluted.

7.3 RESULTS

Inhibition of ^{3}H IdR incorporation in relation to plasma half-life and intracellular deamination

The Ara-C sensitivity test, the plasma half-life and the intracellular deaminating activity in the leukaemic bone marrow cells were determined in 12 AML patients before the start of therapy. Fig 7.1 shows the inhibition percentages plotted versus the plasma half-life values. The inhibition percentage was high (> 70 %) in nine patients with plasma half-life values exceeding 11 minutes. Three patients with plasma half-life values of less than 10 minutes disclosed rather low inhibition percentages (< 65 %). Fig 7.2 shows the inhibition percentages plotted versus the deaminating activities in the leukaemic cells. Although no impressive relation was found, the inhibition percentages tended to decrease at increased deaminating activities. This relation is partly obscured

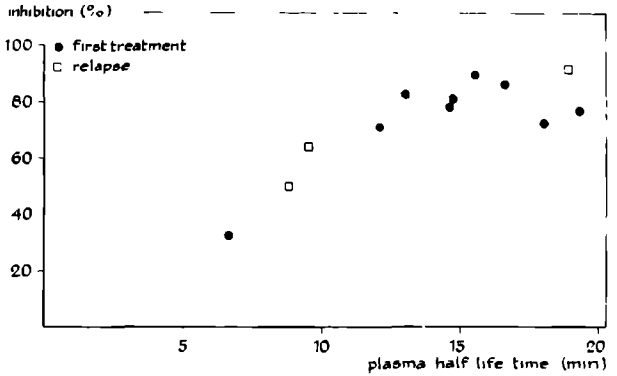

Figure 7.1

The relation between the results of the Ara-C sensitivity test expressed as an inhibition percentage and the plasma half-life values in 12 AML patients.

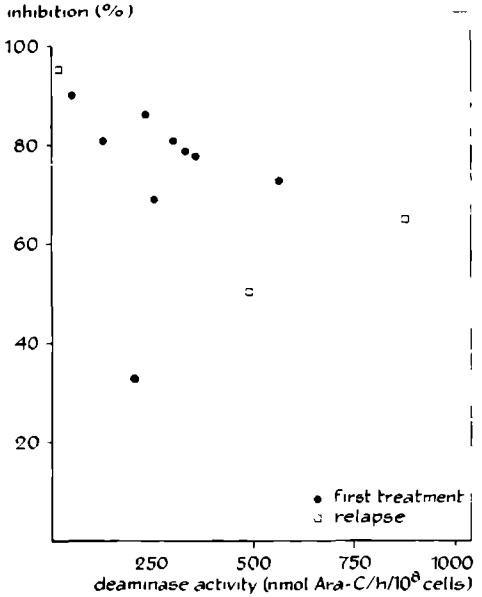

Figure 7.2

The inhibition percentages of the ^{3}HTdR incorporation versus the deaminating activities of the leukaemic bone marrow cells in 12 AML patients.

by the influence of the plasma half-life in the Ara-C sensitivity test (table 7.I). Two out of three patients who were treated for a relapse and were resistant to further therapy showed very high deaminating activities.

Patient Initials	Sex	Age	State of disease	Ara-C treatment		Plasma half life (min)	Inhibition percentage	Deaminase activity nmol/hr 10^8 cells	Result of remission induction
				first course (days)	second course (days)				
L.T.	F	30	Relapse	14		18.9	95	18	C.R.
J.K.	F	43	First	10		15.5	90	48	C.R.
P.S.	M	41	First	14		16.6	86	233	C.R.
M.V.	M	47	First	10		14.7	81	293	+
M.T.	F	38	First	10		13.3	81	126	C.R.
C.A.	F	53	First	14	10	14.6	79	324	C.R.
D.S.	F	23	First	14		14.3	78	342	C.R.
J.H.	M	56	First	10	10	18.0	73	574	C.R.
S.P.	F	19	First	14		11.5	69	247	+
H.H.*	F	28	Relapse	14	10	9.5	65	880	F
J.L.*	F	31	Relapse	10	14	8.8	50	485	F.
A.K.*	M	57	First	10	6	6.6	33	209	P.R.

Table 7.I

In 12 AML patients the relation between the plasma half-life, the deaminating activity, the inhibition percentage of the ^{3}HTdR incorporation and the result of therapy is demonstrated. Patients were subdivided in acute myeloid leukaemia (AML) and acute myelo-monoblastic leukaemia (AMML).

* *These patients were treated with Ara-C starting on day 3 in combination with Adriamycin (day 1) and Vincristine (day 2) during the second course of treatment.*

CR is complete remission; PR is partial remission; F is failure of therapy.

The result of therapy in relation to plasma half-life, intracellular deamination and inhibition of ^{3}HTdR incorporation

The chance of achieving complete remission in acute myeloid leukaemia with Ara-C is positively related to the inhibition percentage in the Ara-C sensitivity test (chapter 6) To investigate the factors which influence the results of the Ara-C sensitivity test, the plasma half-life of Ara-C and the intracellular deaminating activity were measured and weighted against the result of therapy. The patients were treated and evaluated as indicated under "methods". Two patients died during treatment. Ten patients were evaluable (table 7.I).

Complete remission and a high inhibition percentage (I.P. > 70 %) was achieved in seven patients. Four out of five patients with a relatively low deaminating activity (< 300 nmol/h/10^8 cells) came into complete remission after one course of treatment. Two patients with deaminating activities of 324 and 574 nmol/h/10^8 cells needed two courses to achieve complete remission.

A poor bone marrow response and a low inhibition percentage (I.P. < 70 %) was found in three patients. Partial remission was achieved in one patient with a relatively low deaminating activity (< 300 nmol/h/10^8 cells) and a complete failure of therapy was found in the other two patients who showed very high deaminating activities (> 450 nmol/h/10^8 cells).

7 4 DISCUSSION

It is assumed that the inhibition percentage as determined in the Ara-C sensitivity test is related to the amount of Ara-CTP formed in the leukaemic cells (Chou et al., 1975), which in turn depends among other things, on the plasma half-life and the deaminating activities in the cell. The inhibition percentage in relation to these two parameters was studied.

A relation was found between the inhibition percentage and the plasma half-life, while no impressive correlation was found

between the inhibition percentage and the deaminating activities. The inhibition percentages seems to decrease at increasing deaminating activities. These data suggest that the plasma half-life largely determines the extent of the inhibition percentage.
In chapter 6 a relation was found between the inhibition percentage and the result of therapy. Patients with a high inhibition percentage (I.P. > 70 %) came into complete remission, while patients with a low inhibition percentage (I.P. < 70 %) generally failed on therapy.
In this study complete remission associated with a high inhibition percentage (I.P. > 70 %) was found in seven patients. Four out of five of these patients with low deaminating activities (< 300 nmol/h/10^8 cells) came into complete remission after the first course of treatment. The other two patients with high deaminating activities (> 300 nmol/h/10^8 cells) needed two courses to achieve complete remission. The relatively low inhibition percentages in these two patients may be due to the rather high deaminating activities requiring a prolonged treatment.
No complete remission, associated with low inhibition percentages (I.P. < 70 %) was found in three patients with plasma half-life values below 10 minutes. In one of these patients the deaminating activity was low (< 300 nmol/h/10^8 cells) and partial remission was achieved after two courses. After four courses this patient came into complete remission. In the two other patients with high deaminating activities (> 450 nmol/h/10^8 cells) no response to therapy was found.
Despite the small number of patients some preliminary conclusions can be made. The plasma half-life is an important parameter for the chance to achieve a complete remission. The deaminating activity in the leukaemic cells is a second factor of importance for the result of therapy. The plasma half-life as well as the deaminating activity is reflected in the Ara-C sensitivity test.
In patients with plasma half-life values exceeding 13 minutes, the deaminating activity is of minor importance since all these patients came into complete remission after one or two courses of treatment. In patients with half-life values below 10 minutes

the deaminating activity is of more importance since complete remission could be achieved after prolonged treatment in a patient with a rather low deaminating activity, while two others with high deaminating activities were resistant.

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SUMMARY

The cytotoxic drug cytosine arabinoside (Ara-C) is at the moment the drug of choice in the treatment of acute myeloid leukaemia (AML). Unfortunately the results of remission inducing therapy are still disappointing. At most in 60 percent of the patients complete remission is achieved. The failure of treatment may be due to individual differences in pharmacokinetics and metabolism of the cytotoxic drug. This was studied in a number of AML patients as outlined in CHAPTER 1.

In CHAPTER 2 the present knowledge of the metabolism and pharmacology of Ara-C is reviewed. Intracellularly the drug is activated to its triphosphate (Ara-CTP) or deaminated to uracil arabinoside which is non-toxic. Only in the active form the drug inhibits DNA synthesis. The inhibition of DNA synthesis should be more pronounced at higher levels of Ara-CTP which is the resultant of the duration of an effective plasma Ara-C concentration and the net effect of the intracellular enzymatic activities.

To determine the plasma Ara-C concentrations a sensitive bioassay was developed as described in CHAPTER 3. With this assay the plasma concentrations are measured at different time intervals after an intravenous bolus injection. It appears that the plasma Ara-C concentration runs down in a biphasic pattern. From these data the plasma half-life is calculated according to the mathematical analysis of a two compartment open model. It appears that complete remission was attained in patients with a relatively long half-life of Ara-C, while patients with a short plasma half-life failed on therapy as described in CHAPTER 4.

The observed differences in plasma half-life could be due to variations in the amount of drug bound to plasma proteins. To explore this supposition the protein binding of Ara-C was measured in plasma samples and amounted approximately 13 %. From pharmacokinetic studies with other drugs it can be concluded that such a

minor protein binding does not affect the plasma half-life of Ara-C.

As is concluded in CHAPTER 4 the result of treatment in AML depends largely on the plasma half-life of Ara-C. The measurement of plasma Ara-C concentrations is time consuming and not suitable for routine use in clinical situations. To measure the effectivity of Ara-C on the leukaemic bone marrow cells a simple sensitivity test was developed as described in CHAPTER 6. The test is based on the incorporation of tritiated thymidine (^{3}HTdR) in isolated leukaemic bone marrow cells before and one hour after Ara-C administration. The observed difference in ^{3}HTdR incorporation is expressed as an inhibition percentage. It appeared that patients with an inhibition percentage above 70 % came into complete remission after one or two courses with Ara-C. Patients with an inhibition percentage below 70 % in general failed on therapy.

Since the inhibition percentage, as measured in the sensitivity test not only depends on plasma half-life but also on the intracellular deaminating activity the relation between these parameters and the result of therapy was studied (CHAPTER 7). Although the half-life and the result of the Ara-C sensitivity test determine to a great extent the response to treatment (CHAPTER 4 and 6), a difference in the intensity of treatment, needed to achieve complete remission, still exists. Patients with a low deaminating activity achieved complete remission after one course while patients with a high deaminating activity needed two courses of treatment.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 18 april 1945 geboren te Sassenheim. Hij bezocht het Fioretti college te Lisse. Na het behalen van het einddiploma H.B.S.-B begon hij in 1965 met de studie in de geneeskunde aan de Katholieke Universiteit te Nijmegen. In 1968 was hij gedurende een half jaar werkzaam op de afdeling Fysiologie (hoofd: Dr.R.Forster) van de Universiteit van Pennsylvania te Philadelphia. In 1973 legde hij het arts examen af te Nijmegen. Van 1 januari 1974 tot 1 januari 1977 was hij in dienst van de Stichting Koningin Wilhelmina Fonds; de Nederlandse Organisatie voor de Kankerbestrijding.
Sedert 1 januari 1974 is hij in opleiding tot internist aan de Universiteits Kliniek voor Inwendige Ziekten (hoofd: Prof. Dr. C.L.H.Majoor) van het St Radboud Ziekenhuis te Nijmegen.

S T E L L I N G E N

1

De dosering van het cytostaticum cytosine arabinoside dat gebruikt wordt bij de behandeling van patienten met acute myeloide leukemie, dient te worden aangepast aan de eliminatie snelheid.

2

De bevinding dat in muizen de biologische activiteit van het cytostaticum cytosine arabinoside sterk toeneemt na toediening van tetrahydrouridine kan van betekenis zijn voor de behandeling van leukemie patienten, die het cytostaticum snel elimineren.

Neil, G.L., Moxley, T.E., Manak, R.C. (1970) Enhancement by tetrahydrouridine of 1-β-D-Arabinofuranosylcytosine oral activity in L 1210 leukaemic mice. Cancer Res. 30, 2166.

3

De duur van de cytostatische kuren met cytosine arabinoside voor het bereiken van complete remissie bij leukemie patienten dient te worden aangepast aan het percentage prolifererende beenmergcellen.

4

De betekenis van leverscintigrafie voor de stadiëring bij de ziekte van Hodgkin is dubieus.

5

Met de miltkolonie techniek is door Keizer een beschermend effect van sedativa op de stamcellen van met cytostatica behandelende proefdieren aangetoond, dat ook zou kunnen worden verklaard door een verhoogde affiniteit van de stamcellen voor de milt onder invloed van deze sedativa.

H J Keizer. Proefschrift, 1976

6

De toepassing van thrombocyten aggregatie remmers voor de bescherming tegen arteriele vaatocclusies kan mogelijk een klinische betekenis krijgen wanneer de dosering zou worden gerelateerd aan de mate waarin de thrombocytenfunktie wordt geremd

7

De door lithium verhoogde gevoeligheid van menselijke bloedplaatjes voor stoffen die aggregatie induceren blijkt niet primair te berusten op een verhoogde release van ADP.

Imandt, J., Genders, T., Wessels, H., Haanen, C. (1977). Lithium and human platelets. I Its effect on aggregation and release. In the press.

8

Bij verschijnselen van pharyngitis dient de palpatie van de schildklier uitgevoerd te worden.

De Pauw, B.E., De Rooy, H.A.M. (1975). De Quervain's subacute thyroiditis. Neth. J. Med 18, 70.

Gezien de vaak hoge snelheid van tennisballen dient het dragen van een veiligheidsbril voor de netspeler verplicht te worden gesteld.

Nijmegen, 14 januari 1977 H.C. van Prooijen